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"CHARACTERISATION OF LYMPHOCYTE MIGRATION FOLLOWING DNA VACCINATION"

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Abstract

Infection with influenza virus generally produces an immune response in immunocompetent individuals that will protect from further infection of the same strain of virus. The experiments carried out in this study were designed to identify correlates of protection induced by primary experimental influenza virus infection and to compare to/and assess Particle Mediated Immunotherapeutic Delivery (PMID) as a method of DNA immunisation. Initial experiments characterised the protective immune response observed following intranasal influenza virus infection with regard to the humoral and cellular responses generated. It became clear that a combination of systemic and mucosal antibody responses and strong virus-specific $CD8^+$ T cell response in the lungs were important in the control of viral replication.

The data on the immune responses generated following influenza virus infection was used as a "gold standard" to compare the responses observed following PMID immunisation of influenza virus nucleoprotein (NP) DNA. When PMID was assessed for its ability to generate an immune response, a mucosal immune response was observed in the D-NALT as well as a more general systemic response. Whilst a single immunisation induced specific cellular responses, it proved inefficient at generating a humoral response. Protection studies showed that priming by PMID of NP DNA, resulted in a level of protection that reduced viral replication in the lungs by 2 logs.

Investigation into the phenotype of virus-specific T cells generated by either infection or immunisation showed that they have a common $CD44^{hi}CD11a^{hi}CD62L^{lo}$ phenotype in various systemic and mucosal tissues. The cells isolated from the D-NALT displayed an intriguing intermediate level of CD44 expression. *In vivo* migration studies showed a tendency for activated splenic virus-specific $CD8^+$ T cells to home to the D-NALT perhaps implying a role for the D-NALT as a site where effector/memory T cells actively home as part of their role in immune surveillance.

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And finally, I would like to say the biggest thank you of all to my family, Miranda and her family for their support and encouragement over the last few months, and to everyone who asked the question "how's the writing going?"

Thank you!

Dedicated to Mum and Dad

Thank you for your support both emotional and financial
throughout my student days, I am eternally grateful.

My Ain Folk - anonymous

Far frae my hame I wander, but still my thoughts return
To my ain folk ower yonder, in the sheiling by the burn.
I see the cosy ingle, and the mist abune the brae:
And joy and sadness mingle, as I list some auld-warld lay.

And it's oh! but I'm longing for my ain folk,
Tho' they be but lowly, puir and plain folk'
I am far beyond the sea, but my heart will ever be
At home in dear auld Scotland, wi' my ain folk.

0' their absent ane they're telling, the auld folk by the fire:
And I mark the swift tears welling, as the ruddy flame leaps high'r.
How the mither wad caress me were I but by her side:
Now she prays that Heav'n will bless me, tho' the stormy seas divide.

And it's oh! but I'm longing for my ain folk,
Tho' they be but lowly, puir and plain folk'
I am far beyond the sea, but my heart will ever be
At home in dear auld Scotland, wi' my ain folk.

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Abbreviations

AFC	Antibody Forming Cell
APC	Antigen Presenting Cell
BALT	Bronchial Associated Lymphoid Tissue
bps	base pairs
CCL/CXCL	Chemokine Ligand
CCR/CXCR	Chemokine Receptor
CMIS	Common Mucosal Immune System
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DLR	DNA loading rate
DNA	Deoxyribonucleic Acid
D-NALT	Diffuse Nasal Associated Lymphoid Tissue
EID_{50}	Egg infections dose $_{50}$
ELISA	Enzyme linked immunosorbant assay
ELISPOT	Enzyme linked immunospot assay
FCS	Foetal calf serum
GALT	Gut Associated Lymphoid Tissue
HA	Haemagglutinin
HEV	High endothelial venule
ICAM	Intracellular adhesion molecule
IEF	Isoelectric Focusing
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2
IgG3	Immunoglobulin G3

IgM	Immunoglobulin M
IL	Interleukin
i.m.	Intramuscular
IngLN	Inguinal lymph node
LFA-1	Leukocyte function-associated antigen -1 ($\alpha_L\beta_2$)
LP	Lamina Propria
MAb	Monoclonal antibody
MAdCAM	Mucosal addressin cell adhesion molecule
MALT	Mucosal Associated Lymphoid Tissue
MedLN	Mediastinal lymph node
MesLN	Mesenteric lymph node
MHC	Major Hisotcompatibility Complex
min	minutes
NA	Neuraminidase
NP	Nucleoprotein
O-NALT	Organised Nasal Associated Lymphoid Tissue
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pIgR	polymeric immunoglobulin receptor
PMID	Particle Mediated Immunotherapeutic Delivery
PNAd	Peripheral node addressin
PP	Peyer's Patch
pVac	empty plasmid vector
pVac-NP	plasmid vector containing NP gene
PVP	polyvinyl pyrrolidone
RNA	Ribonucleic acid
rpm	revolutions per minute
sec	seconds

sIgA	secretory immunoglobulin A
TBS	Tris Buffered Saline
Tc1/2	T Cytotoxic 1/2
T_{CM}	Central T cell memory
T_{EM}	Effector T cell memory
TCR	T cell receptor
Th1/2	T Helper 1/2
TLR9	Toll-like Receptor 9
URT	Upper Respiratory Tract
VCAM	Vascular cell adhesion molecule
VLA-1	Very late antigen 1 ($\alpha_1\beta_1$ integrin)
VLA-4	Very late antigen 4 ($\alpha_4\beta_1$ integrin)

Chapter 1

Literature Review

1.1 Influenza Virus and Vaccination

1.1.1 Influenza Virus Infection

Influenza is an acute infectious respiratory disease which, in humans, is caused by influenza virus types A and B. The disease usually occurs in epidemic form with an abrupt onset and rapid spread over a geographic region. The virus is transmitted predominantly in virus containing-droplets generated when an infected person talks, coughs or sneezes. The virus gains access to the airways via inhaled aerosols. Transmission is facilitated by enclosed spaces and close proximity to an infected individual. Influenza virus is a highly infectious agent associated with a high rate of transmission.

When influenza virus reaches the airways of a susceptible individual, it starts to infect cells in the lining of the airways. The viral infection spreads through the epithelial cells of the airway linings and causes an inflammation throughout much of the respiratory tract. After an incubation period of 2-3 days, clinical disease becomes evident and can vary from asymptomatic or mild cold-like symptoms to a severe illness and death. Patients with classic "flu-like" symptoms experience a very abrupt onset of symptoms. The illness consists of systemic symptoms including myalgia (muscle ache), headache, fever, chills (shivering), malaise (feeling of illness and discomfort) and anorexia (loss of appetite). Usually the headache and myalgia will predominate. The fever appears at the onset of illness and rises rapidly to 38-40°C. The fever beings to reduce and the symptoms start to resolve within 3-8 days of onset. The patient also initially presents with localised respiratory symptoms which consist of a dry cough, blocked nose, nasal discharge and a sore throat. These symptoms become more obvious as the systemic symptoms subside at around 7 days. It is

not uncommon for patients to feel unwell, lethargic and depressed for some weeks after influenza virus infection.

1.1.2 Epidemiology

In the USA, influenza is responsible on average for 200,000 hospitalisations and approximately 36,000 deaths annually (Centres for Disease Control and Prevention, 2004a). Influenza virus causes disease among all age groups. Rates of infection are highest among children, but rates of serious illness and death are highest among persons aged greater than 65 years and persons of any age who have medical conditions that place them at increased risk for complications from influenza e.g. asthma or other chronic pulmonary diseases. Influenza virus causes annual epidemics, and occasional pandemics. Occasionally, a virus arises which has mutated to overcome the pressure exerted by an immune population. Such mutant or drifted variants may demonstrate the ability to cause new epidemics. However, the real threat results from potential gene reassortments, possibly in an intermediate host, producing a totally new virus containing genes from the animal reservoir (commonly birds and pigs). This viral shift can result in a pandemic influenza virus strain which appears completely foreign to the current immune population.

1.1.3 Influenza Virus Structure

The causative agent of influenza is influenza virus which is a member of the *Orthomyxoviridae* family. Influenza virus is an enveloped virus with a segmented single-stranded negative sense RNA genome. Influenza viruses are divided into three types A, B and C which can be distinguished from each other by antigenic differences between their nucleoprotein (NP) and matrix (M) protein antigens.

Both the influenza A and B viruses are major human pathogens, however the influenza A virus has greater potential to re-assort into more pathogenic strains as it can infect a number of host species. Influenza A virus has a genome comprising eight segments of RNA encoding ten identified polypeptides, nine of which are incorporated into virions (Figure 1). Three of these proteins are inserted into the lipid envelope of the virion, namely haemagglutinin (HA) and neuraminidase (NA) which are involved in cell entry and exit respectively, and the matrix protein M2, an ion channel protein involved in uncoating of the virion. Underlying the membrane is the matrix, or M1 protein, the most abundant protein of the virion, which associates with the ribonucleoprotein (RNP) core of the virus for nuclear export of viral RNA. Also associated with RNPs and M1 protein is the RNA polymerase (comprising the basic polymerases PB1/PB2 and acidic polymerase PA subunits) and single stranded RNA binding protein NP or nucleoprotein. The primary function of NP is to encapsulate the virus genome for the purposes of RNA transcription, replication and packaging. Influenza A virus RNA segment 5 encodes NP which has been found to be relatively well conserved throughout the influenza type A strains with a maximum amino acid difference of 11% (Shu et al., 1993). This makes it an ideal target for vaccine candidates as it should be possible to protect against many influenza viruses using one vaccine.

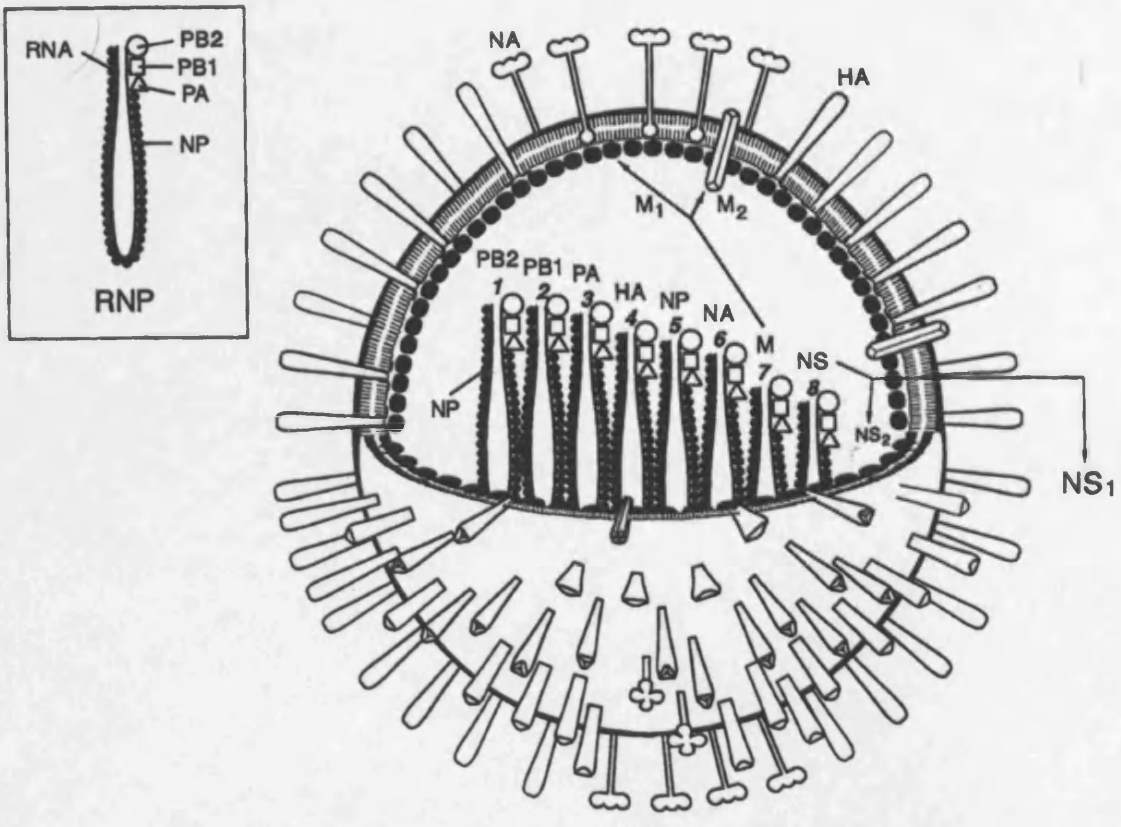


Figure 1: An illustration of the structure of an influenza A virion. Nine proteins are encoded in the genome, 3 of which are found in the membrane, haemagglutinin (HA), neuraminidase (NA) and the matrix protein (M2). The matrix protein M1 underlies the lipid bilayer but also interacts with ribonucleoproteins (RNPs). Within the envelope are eight segments of the single stranded RNA genome contained in the form of an RNP. Associated with RNPs is the RNA transcriptase complex (comprising PB1, PB2 and PA) and nucleocapsid protein (NP). Each of the RNA segments can be seen with the proteins they encode where NS1 and NS2 are non-structural proteins. NS1 is a post-translational regulator with many functions and NS2 associates with M1 and has a role in nuclear export of RNPs

1.1.4 Influenza Virus Replication

Viruses are inert particles that are absolutely dependent on infection of a living host cell for their replication. Influenza virus particles have spikes of HA on their surface which can mediate attachment to host cells via specific receptors (sialic acid). The virus enters the cells and uncoats, removing the protein coat to release the nucleic acid. Transcription and translation of the viral proteins utilises the host cell machinery and new viral particles assemble. The progeny viral particles bud from the host cell. This budding process uses the surface NA to prevent progeny virus from reattaching to the same cell.

1.1.5 The Immunopathogeneis of Influenza Virus Infection

Human influenza A viruses tend to multiply only in the respiratory mucosa, this is due to the requirement of cleavage of the HA of the virus for it to become infectious. Trypsin-like enzymes produced from clara cells found only in the respiratory epithelial perform this cleavage and thus restrict virus growth to the respiratory tract (Kido et al., 1999). Infection generally begins in the upper respiratory tract and spreads to surface epithelium of the lung where disease becomes evident.

$CD8^+$ cytotoxic T cells are believed to play a pivotal role in the clearance of experimental influenza virus infection in mice. While neutralising antibody is also generated later in the response, it does not have a role in clearing primary infection unless the viral load is high (Eichelberger et al., 1991a, Epstein et al., 1998, Graham & Braciale, 1997). Studies using class-1-deficient mice ($\beta 2$ microglobulin$^{-/-}$) which lack $CD8^+$ T cells, have shown that infection with influenza virus A/PR/8/34 leads to increased viral replication and eventual morbidity, indicating that $CD8^+$ T cells are essential for viral clearance (Bender

et al., 1992). $CD8^+$ T cells are first detected in the lung around seven days post infection and their number peaks around ten days post infection, and optimum expansion of the $CD8^+$ T cell subset is dependent on $CD4^+$ T cells (Eichelberger et al., 1991a, Flynn et al., 1998, Riberdy et al., 2000). The accumulation of $CD8^+$ T cells in the lungs results in the clearance of the virus by Fas or perforin mediated mechanisms (Topham et al., 1997). Following viral clearance, there is contraction of the $CD8^+$ T cell response which is independent of the level and duration of antigen presentation (Badovinac et al., 2002). The mediastinal lymph node plays a critical role in generating T cells responses in the lung, but substantial responses are also detected in the spleen, correlating with the distribution of antigen-presenting cells displaying influenza virus antigens there (Flynn et al., 1998, Usherwood et al., 1999b).

Investigations into the role of $CD4^+$ T cells following influenza virus infection initially led to the conclusion that they were not required for the elimination of virus, as CD4-depleted mice could effectively clear influenza virus A/PR/8/34 (Mozdzanowska et al., 2000). A role of CD4 cells in response to influenza virus infection became more apparent when B and helper T cells were absent. B cell-deficient mice treated with anti-CD4 depleting antibodies cannot clear influenza A/PR/8/34 virus and show a high mortality (Mozdzanowska et al., 2000). This data suggests that $CD8^+$ T cells alone cannot clear virus infection and indicates a role for $CD4^+$ T cells in maintenance of a cytotoxic T cell response. $CD4^+$ T cells alone can not effectively clear influenza virus A/PR/8/34 and it is suggested that their main role is the to provide help for antibody production (Mozdzanowska et al., 1997, Topham & Doherty, 1998).

Antibody may play a minor role in primary infection, but what is clear is that neutralising antibody is essential in protecting individuals from further influenza

virus infection (Renegar & Small, 1991a, Renegar & Small, 1991b, Renegar et al., 2004). In particular it is the IgA isotype of virus specific antibody that plays an important role in protection from influenza virus infection and other mucosal pathogens (mechanisms reviewed further in section 1.2.6)

1.1.6 Influenza Virus Mouse Model

Transmission of human influenza virus to laboratory animals was originally demonstrated in ferrets which were inoculated with respiratory secretions from influenza patients and as a result developed febrile respiratory illnesses, which were transmissible among cagemates. It was also observed that mice inoculated under anaesthesia with nasal turbinate material from infected ferrets developed pneumonia (Maher & DeStefano, 2004). These findings led to the use of experimental animals as assay systems for virus infectivity and ultimately to their use as models for assessment of novel antiviral compounds and vaccines. Mice are an ideal animal model to study pathogenesis and the immune response to influenza viruses. They offer the advantages of reproducible responses to infection (especially with inbred strains), small size and easy maintenance.

Influenza virus infection of mice is not associated with a febrile response, in contrast to humans or the ferret model of influenza. The mouse model can represent a severe viral pneumonitis, in which host responses may contribute to the pathogenesis of the disease. This study has focused on the C57BL/6 mouse model which is the best characterised model for influenza A virus infection (Allan et al., 1990, Eichelberger et al., 1991a, Eichelberger et al., 1991b). Influenza A viruses multiply only in the respiratory mucosa; the initial cycles of growth occur in the upper respiratory tract, and the infection later spreads to the surface epithelium of the lung. Viruses are generally eliminated within 10 days of primary

infection, In parallel with the development of $CD8^+$ cytotoxic T lymphocytes (Allan et al., 1990).

1.1.7 Influenza Vaccination – Room for Improvement?

1.1.7.1 Introduction

Current human influenza vaccines contain haemagglutinin as their main or only viral antigen. They have been traditionally produced from viruses grown in embryonated hen eggs, although more recent vaccine stocks have also used viruses grown in tissue culture. These vaccines have contributed to a reduction of the burden imposed on the population by annual influenza epidemics.

Influenza virus haemagglutinin and neuraminidase are large glycoproteins easily accessible on the viral membrane. Both are highly immunogenic and are obvious choices as vaccine antigens. As a result of these proteins being so immunogenic, an infected population can become rapidly protected from reinfection which generates an immunological pressure that can lead to virus mutation and the problems of antigenic drift and/or shift.

The efficacy of the current vaccines depends primarily on the antigenic match between circulating viruses with the strains used for vaccination, as well as the subject's age and immune status. Current vaccines prevent illness in approximately 70-80% of healthy individuals under the age of 65. This number is far lower in the elderly reducing to 30-40% (Palese & Garcia-Sastre, 2002). The ability of current vaccines at preventing death is estimated to be around 80%, even in the higher risk elderly population (Patriarca et al., 1985).

Currently the influenza vaccine is available in two forms either a) inactivated in whole, split and subunit formulations, which are administered intramuscularly or subcutaneously or b) cold-adapted, live attenuated vaccine which is intranasally administered. The inactivated vaccine produces significantly higher serum antibody responses whereas the cold adapted vaccine induces higher levels of nasal IgA (Abramson, 1999, Treanor et al., 1999). The two vaccines have been shown to have similar efficacy in preventing laboratory-confirmed influenza (Treanor et al., 1999). The cold adapted vaccine was shown to be 85% effective and the inactivated vaccine was 71% effective. Live cold-adapted vaccines are a promising approach to immunisation but the response in the elderly is modest. However in combination with the inactivated virus, it provides increased protection from influenza virus infection (Treanor & Betts, 1998).

On October 5th 2004, Chiron Corporation released a press statement announcing that their licence to manufacture Fluvirin® influenza virus vaccine was suspended at its Liverpool facility. This was as a result of concerns of bacterial contamination and failure of Chiron to comply with UK Good Manufacturing Practices. As a result this will prevent release of their vaccine for the 2004-2005 season. Fluvirin® is reported to comprise up to 20% of the influenza vaccine market in the UK (Chiron, 2004). Chiron also supply Fluvirin® to the USA where it takes up approximately 50% of the market share (Centres for Disease Control and Prevention, 2004b). As a result health committees have changed their guidelines for recommending who is to be vaccinated and have requested that healthy individuals aged 2-64 are not vaccinated this year.

1.1.7.2 The Future

The future of influenza vaccine design has focused for some time now on the development of a cold-adapted virus vaccine (Abramson, 1999, Wareing & Tannock, 2001). Virus is grown in cells or embryonated hen eggs and adapted to growth at 25°C. The resulting strains are temperature sensitive with attenuated pathogenicity and are therefore suitable for use as live vaccines. The growth of virus for vaccine use in tissue culture has also become preferable to the use of embryonated hen eggs as a proportion of the population has developed sensitivity to proteins from the eggs.

Efforts have also been made to genetically engineer live viruses to have unique properties that lead to attenuation (Parkin et al., 1997). With the advances in reverse genetics it has also been possible to rescue influenza virus vaccine candidates from cells transfected with plasmids (Neumann et al., 1999).

As a result of the continuing antigenic drift of the virus it is necessary to reformulate the vaccine almost every year. A universal influenza vaccine still remains the goal of many researchers. Some viral proteins are more conserved than others, and several studies have investigated the use of such proteins. The conserved areas of the virus tend to be poorly immunogenic, and are therefore less likely to induce a protective response. Several studies have focused on the M2 matrix protein and have shown promising protection using a murine viral challenge model (Fiers et al., 2004).

1.2 The Mucosal Immune system

1.2.1 Introduction

The mucosal surfaces of the body are characterised as being thin and permeable barriers to the interior of the body and are involved in a variety of physiological activities e.g. gas exchange in the lungs and food absorption in the gut. The necessity for permeability of the surface lining of these sites creates an understandable susceptibility to infection and it is therefore not surprising that the majority of infectious agents invade the human body at these sites.

The mucosal surfaces are similar to the skin in that they act as a barrier and define a boundary. However unlike the skin, the mucosa of the gut and respiratory tract must absorb substances resulting in an inherent susceptibility to infection by invading microbes. The immune system has evolved a specialised capacity to distinguish between food antigens and pathogenic microbes which avoids the potentially harmful induction of a strong immune response to food while retaining the ability to detect and kill pathogenic microbes gaining entry through the gut. The mechanism by which the immune system is able to distinguish between a variety of self and non-self antigens is called tolerance.

1.2.2 Organisation of the Mucosal Immune System

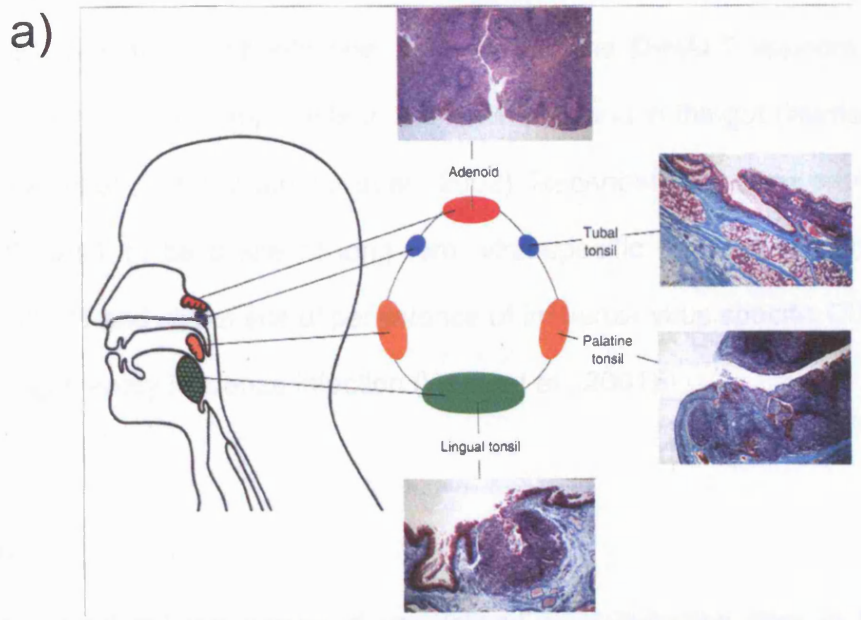
The immune system is divided into two compartments. First is the systemic immune system, comprising bone marrow, the spleen and lymph nodes. The second compartment consists of the lymphoid tissues associated with mucosal surfaces, or mucosal associated lymphoid tissue (MALT), and external secretory glands and is known as the mucosal immune system. Mucosal surfaces are associated with the gastrointestinal, genitourinary and respiratory tracts. The

subepithelial regions of these mucosal surfaces contain an abundance of immunocompetent cells such as B and T lymphocytes. These cells are organised into the MALT and are the main components of the mucosal immune system. Each compartment of the immune system is associated with both humoral and cell mediated responses, however, the nature of the immune response induced in each compartment is different.

Mammals and birds have evolved distinct respiratory and gastrointestinal lymphoid compartments that can participate in the defence of associated distal mucosa. Respiratory lymphoid structures include the bronchial associated lymphoid tissue (BALT), nasal associated lymphoid tissue (NALT), lung parenchyma and alveolar cells in the respiratory tract. Gastrointestinal lymphoid structures constitute the gut associated lymphoid tissue or GALT which comprises the lamina propria (LP), Peyer's patches (PP) and possible solitary lymphoid nodules (SLN) in the intestine (Kraehenbuhl & Neutra, 1992).

1.2.3 The Nasal Associated Lymphoid Tissue

The upper respiratory tract is an important site for defence against invading pathogens as it is the first site of contact of inhaled antigens with the immune system (Kuper et al., 1992). The NALT in the mouse comprises a pair of organised lymphoid aggregates (organised, or O-NALT) located on the posterior side of the palate at the entrance to the nasopharyngeal duct, this is illustrated in Figure 2. It also includes the less organised, diffuse lymphoid tissue lining the nasal passages known as the diffuse or D-NALT (Asanuma et al., 1997). The nasal tissues appear to be functionally equivalent to the Waldeyer's ring of tonsils and adenoids in the human and are most likely to be responsible for the immune response generated following intra-nasal immunisation in the mouse



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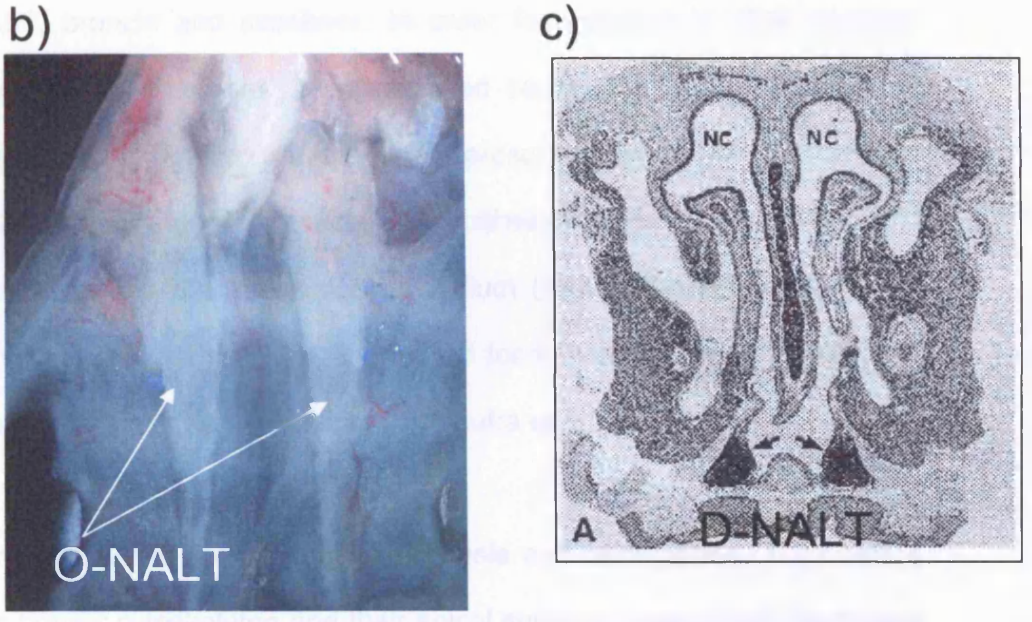


Figure 2: Shows a) the human nasal associated lymphoid tissue also known as the Waldeyer's Ring, comprising the adenoid and various tonsils b) shows the two organised lymphoid structures of the murine organised or O-NALT located on the posterior side of the palate indicated with arrows and c) the diffuse or D-NALT comprising the cells lining the nasal cavity (NC). The O-NALT can also be seen towards the bottom of this figure indicated by arrows.

(Tamura et al., 1998). The O-NALT has been shown to be an inductive site, like the Peyer's patch in the small intestine. By contrast, the D-NALT appears to function as an effector site comparable to the lamina propria in the gut (Heritage et al., 1997, Wu et al., 1997, Zuercher et al., 2002). Recent studies have shown the murine D-NALT to be a site of long term viral-specific humoral immunity (Liang et al., 2001) and also a site of persistence of influenza virus specific $CD8^+$ T cells following primary influenza infection (Wiley et al., 2001).

1.2.4 M Cells

Sampling of luminal antigen occurs at specialised local inductive sites in the organised MALT that appear as single or aggregated mucosal lymphoid follicles in the NALT, bronchi and intestines. In order for antigens to elicit mucosal immune responses, they must be transported across the epithelial barrier at these sites prior to contact with antigen presenting cells (APCs). This is accomplished by virtue of a specialised epithelium over mucosal lymphoid follicles called the follicle associated epithelium (FAE). The FAE contains M cells, a unique epithelial cell type specialised for transport of macromolecules, particles and microbes (Kuper et al., 1992, Neutra et al., 1996).

M cells are well suited for efficient endocytosis and transcytosis. They lack a rigid brush border cytoskeleton and their apical surfaces have broad membrane microdomains which can mediate endocytosis. The M cell basolateral membrane is deeply invaginated to form a large intraepithelial pocket containing T and B lymphocytes and macrophages (Neutra et al., 1996). This structural specialisation brings the basolateral surface to within a few microns of the apical surface and greatly shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. After being transported through M cells, antigens

are processed by macrophages and dendritic cells and presented to T and B lymphocytes present within and below the epithelium.

1.2.5 The Common Mucosal Immune System

In general, mucosal associated lymphoid tissues can be divided into two distinct compartments, namely the inductive and effector sites. Inductive sites are areas where naïve B and T lymphocytes are clonally selected and expand upon antigen contact. The second compartment comprises effector sites where activated B and T lymphocytes relocate after antigen priming in inductive sites to express their effector functions. Previous animal model studies have shown that the inductive sites present in certain locations, such as GALT (PP) or in some specific BALT, function as primary sources of precursor cells which migrate through the lymphatics and blood and ultimately populate remote mucosal tissues (Phillips-Quagliata & Lamm, 1988). More recent studies suggest that such inductive sites are not necessarily restricted to PP. Additional sites have been identified in the nasal mucosa, the palative tonsils and other organised lymphoid tissues of Waldeyer's ring in the nasopharynx (Kuper et al., 1992).

An important consideration for vaccine development is the fact that immunisations at certain inductive sites may give rise to a humoral immune response at multiple effector sites by virtue of the so-called common mucosal immune system (CMIS). An early study on the CMIS demonstrated that following the transfer of Ig-committed B cells from various lymph nodes, the cells demonstrated a tendency to home to certain areas of the mucosal immune system (McDermott & Bienenstock, 1979). Evidence from other studies has indicated that stimulation of the mucosal immune system at one mucosal site can lead to secretory IgA (sIgA) production in the local, as well as distal mucosal

surfaces (Mestecky et al., 1997). For example, antigen stimulation of the PP has resulted in the production of IgA secreting B cells in the intestine, bronchi and the genitourinary tract (Nugent et al., 1998). Other examples include those reported by Dupuy *et al.* (1999) and by Gallichan & Rosenthal (1998) who have shown intranasal immunisation (HPV and HSV vaccines respectively) induced secretory and serum derived humoral responses in the female mouse genital tract.

The concept of the common mucosal immune system is best established for the components of the GALT. PP are inductive sites and have been described as the major locations for antigen specific B cell activation (Craig & Cebra, 1971) and generation of IgA memory B cells (Lebman et al., 1987) as well as for the induction of antigen specific cytotoxic T lymphocytes or CTL (London et al., 1987). Generally, these primed B and T cells relocate from PP, undergo terminal differentiation and eventually home to the lamina propria and the intra epithelial lymphocyte compartment of the gut (McDermott & Bienenstock, 1979). By contrast, little is known about the anatomic location and functional potential of inductive and effector sites in the respiratory tract.

Zuercher *et al.* (2002) analysed the induction of reovirus specific humoral and cellular immune responses in the upper respiratory tract (URT) of mice. They found a number of functional similarities between the MALT of the URT and gastrointestinal tracts. Both sites contain organised secondary lymphoid tissue lined by epithelium, possibly enabling them to effectively absorb antigen. Also, following reovirus infection, both sites result in germinal centre reactions involving the expansion of specific IgA and IgG2a B cells. Both sites also contain a similar draining lymph node that may serve to amplify the responses generated in the PP and NALT respectively. The authors also report detection of

CTL generation in NALT. A five fold higher precursor CTL frequency in NALT compared to lymph nodes that drain the NALT, clearly shows that the NALT is a potent inductive site for specific CTL responses upon intranasal infection.

1.2.6 Antibodies and the Mucosal Immune System

The predominance of IgA over other immunoglobulin isotypes is a characteristic of most mucosal secretions and it is now well established that levels of IgA are much higher in mucosal secretions than in blood. IgA is present in most mucosal secretions in higher concentrations than any other immunoglobulin isotypes, with the exception of the male and female genitourinary tracts where IgG is the prominent isotype (Mestecky et al., 1999).

Production of IgA is especially prominent in the intestinal tract because of its length, surface area and level of exposure to microbial antigens. The existence of a specific receptor-mediated transport mechanism through the epithelium means that most of the IgA produced is secreted locally (Mostov, 1994). A receptor termed the polymeric immunoglobulin receptor (pIgR) binds molecules of immunoglobulin (Ig) that are composed of more than a single basic 4-chain Ig subunit. Such polymeric immunoglobulins include most of the locally synthesised IgA (dimers) and all of the IgM (pentamers).

The pIgR is synthesised by enterocytes and other mucosal epithelial cells and is expressed on their basolateral plasma membrane, where it can bind locally produced IgA and IgM antibodies. The resulting pIgR-immunoglobulin complexes undergo endocytosis and vesicular transport to the apical surface of the enterocytes, where pIgR is proteolytically cleaved at a site between the external and intramembranous domains. This results in the release of IgA, still

bound to the external domain of the pIgR (know as the secretory component), into the mucosal secretions (Lamm, 1998).

Recent studies have shown that some mucosal secretions in humans also contain IgG, where it functions together with secretory IgA (sIgA) in host defence (Robert-Guroff, 2000). Clinical studies have also shown that IgG concentrations predominate over sIgA in the lumen of the lower respiratory tract and female genital tracts (Kitz et al., 2000, Merrill et al., 1985). Spiekermann *et al.* (2002) reported the expression of FcRn, a neonatal MHC class I-related Fc receptor, in bronchial epithelial cells of the adult human, non-human primates and the mouse. Their data defines a function of FcRn at mucosal surfaces and explains how IgG can cross epithelial barriers by receptor mediated transcytosis and can also be reabsorbed by epithelial cell surface expression of FcRn to transport luminal IgG-bound antigen for processing in the LP or systemically.

It is recognised that IgA, and more recently IgG, present in intestinal and other mucosal secretions, provide a first line of immunological defence against microbial pathogens by helping to prevent them from adhering to and penetrating the mucosal epithelium (Lamm, 1997, Robert-Guroff, 2000). IgA has also been shown to be able to mediate effective resistance to microbial challenge in mice (Mazanec et al., 1987, Michetti et al., 1992). The production of Sendai and influenza virus was specifically inhibited by the antibody (Mazanec et al., 1995) and systemic administration to mice of an IgA-class monoclonal antibody, with specificity for rotavirus inner capsid protein, is able to inhibit intestinal epithelial infections (Burns et al., 1996).

Evidence has emerged in support of a role for IgA in protection inside mucosal epithelial cells. This possibly arises from the obligatory pIgR-mediated

transepithelial route IgA follows to enter the secretions. The initial study supporting this idea was carried out in two chambered culture vessels containing polarised monolayers of epithelial cells that expressed pIgR on the basolateral surface. The cells were infected from the apical surface with a virus and IgA-class monoclonal antibody against viral envelope protein was added below the basolateral surface, from which it could be endocytosed via the pIgR and transcytosed. Under these conditions, the production of Sendai and influenza virus was specifically inhibited by IgA (Mazanec et al., 1992, Mazanec et al., 1995).

1.2.7 The Mucosal Immune System and its Cellular Repertoire

In addition to the organised lymphoid tissue, in which induction of immune responses occur within the mucosal immune system, small foci of lymphocytes and plasma cells are scattered widely throughout the lamina propria of the gut wall. These represent the effector cells of the gut mucosal immune system.

The T lymphocytes of the gut can be divided into a number of groups. There are the conventional $\alpha\beta$TCR (T cell receptor) CD4 and CD8 cells, but there are also those with more unusual surface phenotypes such as, $\gamma\delta$TCR and $\alpha\beta$TCR, CD8$\alpha\alpha$ lymphocytes. The receptors of this second class of T cells do not bind to normal MHC:peptide complexes but instead bind to a number of different ligands including MHC class 1b molecules (Steele et al., 2000).

Although $\gamma\delta$ T cells are well represented in peripheral blood and in afferent and efferent lymph, they are rarely found in lymph node parenchyma, spleen, PP or thymus (Bucy et al., 1988, Hein & Mackay, 1991). $\gamma\delta$ T cells are localised in tissues (Hein & Mackay, 1991) and are disproportionately abundant in the

intestine, and are commonly found as intraepithelial lymphocytes (IELs) interspersed between enterocytes (Goodman & Lefrancois, 1988). Analysis of the respiratory epithelium has shown no significant levels of $\gamma\delta$ T cells or $\alpha\beta$TCR, CD8$\alpha\alpha$ lymphocytes (Liang, 2001).

The lymph nodes and T cell areas of the spleen are the only known anatomical structures in which great diversity of antigen can be presented and the resulting clonal expansion and differentiation of lymphocytes supported. Therefore, the relative absence of $\gamma\delta$ T cells from these areas is consistent with the hypothesis that $\gamma\delta$ T cells do not routinely rely on professional antigen presenting cells (APCs) for antigen recognition. Instead they may recognise antigen directly in tissues, sometimes using extremely limited TCR $\gamma\delta$ diversity (Janeway et al., 1988). Janeway *et al.* also propose that in addition to recognition of a diversity of microbial antigens $\gamma\delta$ T cells can also respond to unique "stress antigens" which are markers of cell infection or transformation.

$\gamma\delta$ T cells have also been implicated in autoantibody production and development (Peng et al., 1998). They may also be beneficial during transplantation (Gorczynski et al., 1996). These authors reported that down-regulation of $\alpha\beta$ T cells correlated with the prolongation of allogenic graft survival, which is thought to be mediated by the $\gamma\delta$ T cell suppression of IL-12 and IFNγ production by mesenteric lymph node T_H1 cells. Finally $\gamma\delta$ cells have been implicated in regulation of T cell leukaemias (Penninger et al., 1995).

1.2.8 Effector Mechanisms Against Mucosal Infection

The mucosal immune system is structurally and functionally divided into sites for antigen uptake and processing or inductive sites, and effector sites engaging

effector cells including lymphocytes, granulocytes and mast cells. The best characterised example of inductive and effector sites in the mucosal immune system is that of the Peyer's patch and lamina propria of the gut (Brandtzaeg et al., 1999, Hein, 1999).

The major mucosal effector mechanisms to respiratory virus infection have been described in section 1.1.5 but in addition to respiratory mucosal effector mechanisms there are also mechanisms which are unique to the gut mucosa. Like the respiratory mucosa, the GALT is characterised by the presence of specialised M cells within the FAE (as discussed in section 1.2.4). The GALT is also characterised by the presence of intraepithelial T cells with unusual surface phenotypes for example the $\gamma\delta$TCR or the CD8$\alpha\alpha$ molecule (as discussed in section 1.2.7). Intraepithelial lymphocytes (IELs) are distinct from systemic T cells with their unusual surface phenotype and can be divided into 2 categories: type a IELs with the conventional $\alpha\beta$TCR which recognise antigen presented by MHC, and type b IELs which include $\gamma\delta$TCR T cells and are not restricted by conventional MHC. Type b IELs are cytolytic and secrete cytokines and chemokines (Barrett et al., 1992, Taguchi et al., 1991), they have also been shown to protect from infection by defending the integrity of the epithelial barrier to infection by promoting epithelial wound healing, maturation and general homeostasis of the gut (Boismenu & Havran, 1994, Komano et al., 1995). The gut is also specialised in that IgA is the predominant immunoglobulin isotype produced. This has already been reviewed in section 1.2.6.

1.3 The Mucosal Immune System and Vaccination

1.3.1 Introduction

The respiratory tract is the first site of attack by many major pathogens including influenza virus. Effective generation of immune responses at the respiratory mucosa and regional lymphoid tissues is likely to be of great importance for the prevention of infectious diseases. However, the majority of vaccines currently used to induce protection from infectious respiratory diseases work by activating the systemic immune system. Although the cells of the immune system may circulate between mucosal and non-mucosal tissues after activation, vaccination focused on the direct activation of the mucosal immune system may improve overall efficacy.

Numerous studies have indicated that induction of systemic immunity through parenteral immunisation can effectively protect against systemic infections but usually fails to specifically protect the mucosa surfaces (Mestecky, 1987). Induction of mucosal immunity at the site of infections on the other hand, provides the main protection against mucosal infection (Mestecky & McGhee, 1992). The mucosal immune system differs in several other ways from the systemic immune system. Mucosal immunisation frequently results in the stimulation of both mucosal and systemic immune responses, while systemic immunisation typically only induces systemic responses without activating the mucosal immune system.

Mucosal surfaces such as the gastrointestinal, respiratory and genital tracts, are the principal sites of entry and colonisation for many pathogens. In order to effectively protect these surfaces, the mucosal immune system needs to be properly activated through direct mucosal immunisation (Chen, 2000). Mucosal

immunisation potentially offers many advantages over parenteral immunisations. There is an obvious advantage of having an orally or nasally administered vaccine over the use of a conventional needle and syringe to administer a vaccine. Mucosal delivery may enhance vaccine efficacy by inducing both mucosal and systemic immunity simultaneously but it also has the potential to increase vaccine safety and minimise vaccine adverse effects by avoiding direct contact between potentially toxic vaccine components and the systemic circulation. It may also reduce the need for trained personnel required for administration. Finally, it allows for easy administration of multivalent vaccines (Lee et al., 2000).

1.3.2 Approaches to Developing Mucosal Vaccines

The various barriers of the mucosal immune system prevent efficient absorption of mucosally delivered vaccines. The barriers include mechanical barriers like mucus that clears antigens from the respiratory system and enzyme degradation. Mucosal immunisation also has to overcome the acquired barrier of tolerance, whereby the immune system can have reduced capacity to develop an immune response upon re-exposure to or immunisation with the same antigen. Oral tolerance is an important natural mechanism by which we avoid developing allergic reactions to ingested foods and to certain other antigens.

There are various approaches to developing mucosal vaccines including using live microbes as vectors for delivery or encapsulation of the vaccine to protect it from the environment that it enters. Recombinant viral and bacterial vectors have been created to serve as a vehicle to transport and express foreign antigen. These vectors are attenuated through mutation while retaining the ability to invade and populate a mucosal surface. There is also the possibility

that several different antigens can be expressed from one live vector, potentially achieving protection against a number of diseases with a single immunisation (Lee et al., 2000). Recombinant adenoviruses that express Simian immunodeficiency virus (SIV) envelope proteins have been cloned and administered via either intranasal or oral routes to Rhesus macaques. These viral vectors have induced strong humoral, cellular and mucosal responses in these primates (Buge et al., 1997). Adenoviruses have also been engineered to express bovine herpes simplex virus glycoproteins and have been shown to stimulate high levels of glycoprotein D specific IgA (Papp et al., 1999). Finally, Salmonella has also been investigated for suitability for use as a live vector (Nayak et al., 1998). Nayak *et al.* demonstrated that expression of pneumococcal surface protein A in Salmonella resulted in resistance to *S. pneumoniae* in mice with stimulation of systemic and mucosal humoral immune responses demonstrated.

Current efforts in developing mucosal vaccines are directed towards finding more efficient means of delivering antigens to the mucosal immune system that bypass the phenomenon of tolerance. There is also great emphasis on research focusing on the discovery of effective, safe mucosal adjuvants that generate stronger mucosal immune responses.

1.4 DNA Vaccination

1.4.1 Introduction

DNA immunisation arose from the discovery that injections of pure plasmid DNA encoding B-galactosidase, luciferase or chloramphenicol acetyltransferase resulted in long term reporter gene expression in transfected muscle fibres

(Wolff et al., 1990). It was subsequently shown that if the plasmid encoded an antigenic protein, it was possible to induce immune responses against the expressed protein (Tang et al., 1992). Tang *et al.* described the production of antibodies against human growth hormone induced by gene gun immunisation of DNA coated gold microparticles. Gene gun plasmid delivery was also shown to elicit cell mediated immunity leading to cross strain protection against influenza virus infection (Ulmer et al., 1993b). Antibody responses and/or protection were also shown to be induced by DNA vaccination against several other viral antigens, including HIV (Wang et al., 1993), a lethal influenza challenge (Robinson et al., 1993) and rabies virus (Xiang et al., 1994).

An advantage for expressing antigen in the host rather than having to administer antigen (such as inactivated virus, recombinant proteins or peptides) is that the potential for losing antigenicity during any inactivation process is avoided. Expressing the antigen in the host also allows synthesis of proteins with a conformation and post translational modifications that are likely to be similar to or identical to the native antigen. Finally, intracellular antigen processing and presentation by MHC I can lead to the induction of CTL responses.

1.4.2 Immune Responses Induced by DNA Vaccines

DNA vaccines have been shown to induce high-titre serum antibodies to a variety of different antigens including viral, bacterial, eukaryotic and tumour-associated proteins. For example, DNA constructs encoding viral proteins including influenza virus haemagglutinin (HA) can induce a long-term humoral response *in vivo* (Fynan et al., 1993, Ulmer et al., 1994). HA contains a signal sequence that allows transport to the cell surface via a secretory pathway, but other proteins not destined for secretion such as influenza virus NP have also

been shown to have the capacity to induce long-lived humoral responses in mice, with antibody responses persisting from six months to two years (Ulmer et al., 1993a, Yankauckas et al., 1993).

The ability of DNA vaccines to induce cytotoxic T-cell (CTL) responses was first demonstrated using influenza virus NP (Ulmer et al., 1993a). This antigen is a conserved, internal protein of the virus and a target for cross-reacting CTL (Wraith et al., 1987). Intramuscular injection of a plasmid coding for influenza virus NP was found to induce CTL in mice and furthermore these mice were observed to be protected from a cross strain, lethal challenge of influenza virus (Ulmer et al., 1994, Ulmer et al., 1993a).

The precise mechanisms by which CTL are induced following DNA vaccination are still unclear. However, there are three proposed mechanisms by which a CTL response may be induced. Firstly, and least convincingly, it is possible that myocytes are directly transfected after intramuscular injection. However, the low level of expression of MHC I and the lack of detectable levels of co-stimulatory molecules by muscle cells suggests they may not be capable of functioning as professional APCs. The second idea is that of direct priming, where APCs resident near the site of immunisation become directly transfected with plasmid DNA, which can in turn present peptide via the MHC class I pathway to elicit a CTL response. Finally, and most convincingly, is the cross presentation hypothesis where it is proposed that antigen is transferred from transfected monocytes to professional APCs (Corr et al., 1999, Ulmer et al., 1996). This idea of cross-presentation is supported by a study which investigated responses elicited by intramuscular injection of DNA encoding either secreted or cytoplasmic forms of an antigen. The secreted form of the antigen proved to generate stronger CTL responses and supports the facilitation of cross-

presentation of the antigen from non-professional APCs (Boyle et al., 1997). Recent data has also shown evidence that cross priming is the predominant mechanism for inducing $CD8^+$ T cell responses following gene gun DNA immunisation (Cho et al., 2001). This study reported that professional APCs are capable of taking up exogenous antigen from DNA transfected non-APCs such as monocytes and keratinocytes.

DNA vaccination has been shown to generate a variety of immune responses depending on the delivery technique (Oran & Robinson, 2003, Oran & Robinson, 2004). Studies using DNA vaccines expressing influenza virus HA in either a membrane bound, or secreted form, compared delivery by intramuscular injection and gene gun immunisation. Typical strong Th1 type responses were observed following intramuscular immunisation of DNA encoding membrane bound HA. The strongest type 2 responses were observed following gene gun immunisation with the secretory form of HA. Further investigation following viral challenge revealed that regardless of the T-helper/T-cytotoxic status, Tc1 type cells underwent more efficient expansion, and only Tc1 cells were found at the site of challenge. It was concluded that the type of memory T cell response induced by DNA vaccination, be it Th1/Th2 or Tc1/Tc2, does not influence the response at the site of an infection.

DNA vaccines have other advantages over traditional whole microbial vaccines and subunit vaccines. One advantage is they do not require the use of purified proteins, which can be difficult and expensive to produce and store in large quantities, or viral vectors which may be associated with safety issues. More importantly, vaccines that use inactivated microbes or their components do not provide endogenously synthesised proteins and generally act as poor agents for eliciting CTL responses, which are considered to be important for controlling

most infections. DNA-mediated expression of the immunising proteins in host cells results in the presentation of normally processed protein to the immune system which is important for inducing immune responses against the native forms of proteins (Webster et al., 1994).

1.4.3 DNA Delivery Methods

The immune response can be dramatically altered depending on the delivery route of DNA immunisation chosen. In the past, the most common route of immunisation has been direct intramuscular injection. Of the original DNA immunisation studies, this was the method of choice and, as previously described, has proven to be a good method of inducing both humoral and cellular immune responses. More recently, other methods of DNA delivery have been investigated with gene gun, epidermal powder immunisation and biojector being three examples. The biojector is a needle free device for delivering substances intramuscularly or subcutaneously.

A recent study compared the $CD8^+$ T cell responses generated following DNA delivery by syringe, gene gun, and biojector (Trimble et al., 2003). It was shown that DNA vaccine administered via gene gun generated the greatest frequency of antigen specific $CD8^+$ T cells compared to the other methods. Gene gun administration also proved to be favourable because the dose of DNA required to generate the response was much lower than for the other methods. It is thought that the superior response observed using the gene gun is due to the ability of this route of administration to generate professional APCs that express the antigen as a result of a low number of APCs being directly transfected. Intradermal immunisation via the gene gun can directly target antigen to

Langerhans cells, which are immature DCs, but nevertheless professional APCs (Condon et al., 1996).

Another method of DNA delivery recently investigated is the encapsulation of DNA in lipids to protect the nucleic acid from degradation following mucosal immunisation. Intranasal immunisation of DNA-lipid complexes expressing a model antigen was found to result in an increase in production of antigen specific IgA in murine vaginal and rectal tracts, while an antigen specific CTL response was observed in the spleen, genital and cervical lymph nodes (Klavinskis et al., 1999). These results show potential for application to immunisation against any pathogen invading at a mucosal site.

1.4.4 Particle Mediated Immunotherapeutic Delivery (PMID)

The gene gun is a ballistic device designed to propel DNA coated gold microparticles into the epidermis or other tissues. PMID of DNA to the epidermis has resulted in immunity to pathogens that invade mucosal surfaces, including influenza virus and rotavirus (Chen et al., 1997, Ulmer et al., 1993a, Yankauckas et al., 1993), and it is possible that epidermal inoculation is as effective at inducing immunity at distal mucosal sites as that obtained by direct mucosal immunisation. Protection from rotavirus infection elicited by gene gun delivery to perianal tissue was recently compared to that elicited by conventional epidermal gene gun immunisation (on the abdomen) (Chen et al., 1999). The protection obtained by mucosal immunisation was higher than that for the same amount of DNA administered by epidermal immunisation. This result is consistent with the hypothesis that direct mucosal immunisation is a more effective means of generating protective immunity against mucosal pathogens.

1.4.5 Enhancing the Immune Response to DNA

Bacterial plasmid DNA contains immunostimulatory sequences (ISS) which exhibit adjuvant properties. The immunostimulatory properties were first identified by a study focusing on why vaccination with Bacille Calmette-Guérin (BCG) reduced tumour growth (Tokunaga et al., 1999). Further research mapped the stimulating sequence of BCG and subsequently extended these observations to other bacterial DNA. The stimulatory potential of prokaryotic DNA was later found to relate to the presence of unmethylated CpG motifs (Krieg et al., 1995) and since then CpG motifs on bacterial DNA have been shown to have adjuvant properties (Roman et al., 1997). CpG motifs have subsequently been shown to directly activate macrophages and dendritic cells (Sparwasser et al., 1998, Sparwasser et al., 1997) and recognition of bacterial CpG has been demonstrated to be mediated by a Toll Like Receptor 9 (TLR9). This was confirmed by a report that TLR9 deficient mice did not respond to immunostimulatory CpG-DNA (Hemmi et al., 2000).

Several studies have investigated whether immune responses can be enhanced if cellular uptake of DNA following needle injection is a limiting factor. Electroporation has been used as a technique to enhance the cellular uptake of DNA (Widera et al., 2000). The authors demonstrated that electroporation results in increased delivery of DNA to cells with subsequent increased expression of antigen and superior humoral immune responses against weakly immunogenic antigens.

Research has also focused on co-administration of plasmids expressing cytokines which are known to emphasise certain components of immune defence that correlate with protection. One such example combined mucosal

immunisation with co-administration of cytokine genes. Intranasal delivery of a combination of an HIV DNA vaccine candidate with plasmids expressing IL-12 and GM-CSF was shown to induce a strong CTL response to the HIV antigen both systemically and more importantly at mucosal sites (Okada et al., 1997). Other more recent examples have shown protection against HSV following co-administration of plasmids expressing IL-12 and IL-18 (Lee et al., 2003), and the generation of stronger memory responses following vaccination and co-administration of a plasmid expressing IL-12 (Chattergoon et al., 2004).

Another approach to enhance the immune responses elicited by DNA has been to deliver the DNA mucosally and directly to M cells (Wu et al., 2001). This study focused on the coupling of DNA to reovirus protein sigma 1, which when coupled to polylysine could directly bind to the apical surface of M cells in the NALT. Intranasal immunisation with this complex resulted in antigen-specific serum IgG and long-lived mucosal IgA as well as a substantial CTL response.

1.4.6 DNA Vaccines and Influenza

Influenza DNA vaccines have been extensively studied and constructs encoding the surface glycoproteins, HA and NA, internal proteins NP and M1 and non structural protein NS1 have all been assessed for their ability to generate protective immune responses (Chen et al., 1998, Robinson et al., 1997, Ulmer et al., 1993a). Immunisation with plasmid DNA encoding influenza HA is capable of inducing cell-mediated and humoral immunity and protecting against intranasal challenge with influenza virus (Ulmer et al., 1994, Webster et al., 1994). Furthermore, induction of CTLs and protection against virus challenge has been demonstrated with plasmid DNA encoding influenza virus NP (Ulmer et al., 1993b). These studies have primarily been carried out by introducing DNA by

intramuscular injection, and research into gene gun immunisation has been limited and less productive (Chen et al., 2000).

1.5 Lymphocyte Trafficking

1.5.1 Introduction

In 1964, Gowans and Knight demonstrated that circulating naïve lymphocytes enter secondary lymphoid tissue via high endothelial venules (HEV) before subsequently returning to the circulation through efferent lymph (Gowans & Knight, 1964). Further research showed how memory T lymphocytes selectively recirculate back through tissues from which they originated (Cahill et al., 1977) and together these early studies began to contribute to the hypothesis of tissue specific lymphocyte recirculation. The benefits of recirculation are two-fold; firstly, antigen specific lymphocytes have the chance to come into contact with specific antigen regardless of where the antigen is in the body. Secondly, it ensures that particular subsets of lymphocytes are delivered to where they are required the most.

Recirculation begins with leukocytes interacting transiently and reversibly with vascular endothelium through adhesion receptors in a process called rolling. This is the first step in a multistep model of leukocyte recruitment (Butcher, 1991, Springer, 1994). As a leukocyte rolls along the surface of the endothelium it is allowed to sample the surface for activating factors. Activation allows a firmer adhesion between the leukocyte and the endothelium before the leukocyte is slowed down completely due to high affinity interactions. At this stage leukocytes can receive further signals to trigger their transmigration through junctions between cells in the endothelial layer. Once they have entered

a tissue, leukocytes migrate along chemotactic gradients to an area of inflammation. At this point they can exert their effect before either dying or leaving a tissue and returning to the circulation via the efferent lymph and thoracic duct.

1.5.2 Chemokines

Chemokines are a group of small, structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G protein-coupled receptors. Chemokines have been subdivided into 2 major groups on the basis of the arrangement of their two N-terminal cysteine residues, CXC and CC, depending on whether the first two cysteine residues have an amino acid between them (CXC) or are adjacent to each other (CC).

Chemokines can be divided into two effector categories: homeostatic and inflammatory. Homeostatic chemokines are expressed in certain tissues, suggesting a specific function involved in cell migration. The inflammatory chemokines, however, are strongly induced by inflammatory or immune stimuli and participate in the development of immune/inflammatory reactions. Recently, chemokines have been shown to exert pivotal roles in the development and function of the immune system. Two studies have shown crucial roles for the chemokines CXCL12 and CCL3 in B cell and T cell development (Kelner & Zlotnik, 1995, Nagasawa et al., 1996).

1.5.3 The Multistep Model of Leukocyte Recruitment

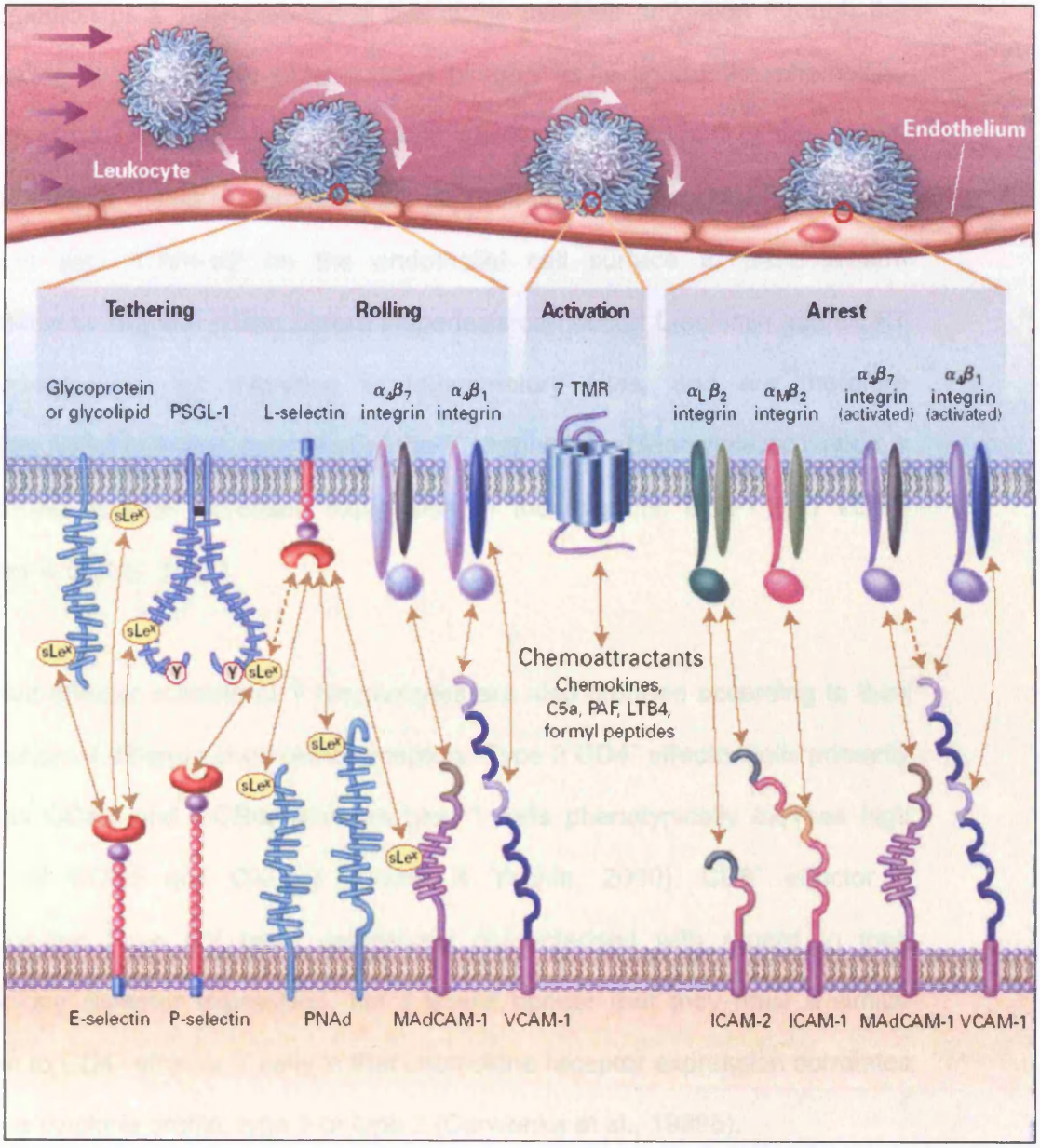
All leukocytes are believed to extravasate through a series of steps that are considered to be similar regardless of the type of leukocyte involved (Butcher, 1991). The multistep model can be seen in Figure 3. The initial, reversible interaction is mediated by the interaction of selectins with their glycoprotein ligands. The selectin family comprises three members which fall into two groups: The first is L-selectin (CD62L) which is expressed on many leukocytes including naïve T cells. The second group includes E-selectin (CD62E) and P-selectin (CD62P) both of which are expressed by activated endothelium. All selectins bind sialomucins that present oligosaccharides related to sialyl-Lewis^x. L-selectin recognises sulphated sialyl-Lewis^x-like sugars called peripheral-node addressin (PNAd) (Vestweber & Blanks, 1999). Selectin-mediated bonds are very weak and the pressure exerted by blood flow can easily dissociate upstream bonds, while new bonds form downstream. This selectin-glycoprotein interaction causes the leukocyte to roll across the surface of the endothelium and results in the loose tethering of the leukocyte allowing it to interact further with the endothelium.

The second step occurs as the leukocyte is rolling across the surface of the endothelium, where it can become activated by chemokines which are involved in the stimulation of leukocyte movement. In response to chemokines, rolling leukocytes subsequently rearrange their cytoskeletons and change in shape from spherical to a more flattened shape which allows integrins on the cell surface to engage with their ligands on the endothelial cells and further slow the movement of the leukocyte. The most common integrins involved in this second stage of adhesion are leukocyte function-associated antigen type 1 (LFA-1; also referred to as CD11aCD18 and $\alpha_L\beta_2$ integrin) and the two α4 integrins, $\alpha_4\beta_1$

(also know as very late antigen-4 or VLA-4) and $\alpha_4\beta_7$ (also known as LPAM-1). The α_4 integrins have also been shown to play a part in the rolling step of the model (Berlin et al., 1995). Leukocytes demonstrate high affinity binding to endothelial cells when the interaction is mediated by integrins. Integrins recognise specific ligands on the endothelial cell surface. The ligands include intracellular adhesion molecules ICAM-1 / ICAM-2 (which bind LFA-1) and vascular cell adhesion molecule (VCAM-1) as well as mucosal addressin cell adhesion molecule (MAdCAM-1) which function as the ligands for the α_4 integrins. Leukocytes now exhibit firm adherence, they no longer exhibit rolling and appear fixed. Studies on blood lymphocytes have shown that they only stop if their $\beta 2$ integrins (e.g. LFA-1) are triggered into a high affinity conformation by signalling through G protein coupled receptors (Bargatze et al., 1995, Warnock et al., 1998). More recently, studies on the migration of T lymphocytes have shown that the chemokine CCL21 (also known as SLC-secondary lymphocyte chemoattractant and thymus-derived chemotactic agent TCA-4) triggers LFA-1-mediated arrest of rolling T lymphocytes (Campbell et al., 1998b, Stein et al., 2000, Warnock et al., 1998). Chemokines have been shown to be capable of mediating this arrest process on peripheral blood lymphocytes and lymph node cells (Campbell et al., 1998b).

The final step in the model is the transmigration of leukocytes through the vessel wall between the endothelial cell junctions and into the tissue. Once through the endothelial layer, leukocytes can migrate along gradients of chemoattractants towards a site of inflammation.

Figure 3: An illustration of the multistep model of leukocyte migration taken from von Andrian, U. H. & Mackay, C. R. (2000). T-cell function and migration. Two sides of the same coin. *N Engl J Med* **343**, 1020-34. Graduated arrows at the top of the figure demonstrate the flow of blood in the vessel and how the velocity of blood is fastest at the centre of the vessel and approaches zero at the epithelial surface. Leukocytes interact transiently and reversibly with vascular endothelium through adhesion receptors in a process called rolling. Tethering and rolling is facilitated by selectins and the α_4 integrins. As a leukocyte rolls along the surface of the endothelium it is allowed to sample the surface for activating factors. Activation occurs via interactions with chemoattractants bound to seven transmembrane domain receptors on the endothelial cells. Activation allows a firmer adhesion between the leukocyte and the endothelium via β_2 and α_4 integrins before the leukocyte is slowed down completely due to high affinity interactions. At this stage leukocytes can receive further signals to trigger their transmigration through junctions between cells in the endothelial layer.


Leukocyte
Endothelium
Tethering
Rolling
Activation
Arrest
Glycoprotein or glycolipid
PSGL-1
L-selectin
$\alpha_4\beta_7$ integrin
$\alpha_4\beta_1$ integrin
7 TMR
$\alpha_L\beta_2$ integrin
$\alpha_M\beta_2$ integrin
$\alpha_4\beta_7$ integrin (activated)
$\alpha_4\beta_1$ integrin (activated)
sLeˣ
Y
Chemoattractants
Chemokines, C5a, PAF, LTB4, formyl peptides
E-selectin
P-selectin
PNAd
MAdCAM-1
VCAM-1
ICAM-2
ICAM-1
MAdCAM-1
VCAM-1

1.5.3.1 T Lymphocytes and the Multistep Model

Naïve T lymphocytes gain access to the lymph nodes through HEVs. Expression of L-selectin on the lymphocyte is essential for binding to PNAd and the initial contact to the HEV (Arbones et al., 1994). Furthermore, successful extravasation of a T lymphocyte at this stage involves activation through the chemokine CCL21 (Gunn et al., 1999) binding to its ligand the chemokine receptor CCR7 on the lymphocyte (Campbell et al., 1998a, Yoshida et al., 1998). Finally, naïve T lymphocytes express the β_2 integrin LFA-1 which interacts with ICAM-1/2 on the endothelial cell surface to promote firm adherence to stop the rolling before diapedesis can occur. L-selectin and CCR7 are unnecessary for migration to inflammatory sites, and are therefore downregulated following activation of the T lymphocyte. Meanwhile activation is associated with an increased expression of the integrins LFA-1 and VLA-4 (Mobley & Dailey, 1992).

Different effector subsets of T lymphocytes are also grouped according to their expression of different chemokine receptors. Type 2 $CD4^+$ effector cells primarily express CCR3 and CCR4, whereas type 1 cells phenotypically express high levels of CCR5 and CXCR3 (Zlotnik & Yoshie, 2000). $CD8^+$ effector T lymphocytes have not been extensively characterised with regard to their chemokine receptor expression, but it would appear that they have a similar pattern to $CD4^+$ effector T cells in that chemokine receptor expression correlates with the cytokine profile, type 1 or type 2 (Cerwenka et al., 1999b).

1.5.3.2 Integrins versus Selectins

It has been shown that both integrins and selectins play important roles in cellular trafficking. However, integrins have been shown to play a more important role when the leukocytes are activated $CD8^+$ effector lymphocytes. A study was recently carried out to investigate the functional impact of blocking the effects of certain integrins and selectins on the migration of effector $CD8^+$ T cells to sites of viral replication (Bartholdy et al., 2000). This investigation blocked either; two of the major selectins (E and P-selectin), or one selectin (P) and one integrin (ICAM-1) or finally two integrins (ICAM-1 and VCAM-2). Results showed that failure to interact with endothelial selectins is of little functional relevance; however, integrins are crucial for a virus-induced T cell mediated inflammatory response.

1.5.4 Migration of Effector T Lymphocytes

Naïve T lymphocytes express high levels of L-selectin, low levels of LFA-1 and are CCR7 positive, all of which aid the extravasation of these cells at HEVs to enter lymph nodes (Warnock et al., 1998, Weninger et al., 2001). Upon activation, these naïve lymphocytes down-regulate these lymph node homing molecules and enhance expression of molecules involved in the migration to non-lymphoid tissues such as the skin and intestinal mucosa. For example, a subset of circulating memory $CD4^+$ and $CD8^+$ T lymphocytes express E and P-selectin ligands in addition to the chemokine receptor CCR4. Expression of this combination of surface antigens is a phenotype known to be characteristic of skin homing lymphocytes (Campbell et al., 1999, Tietz et al., 1998, Xie et al., 1999). By contrast, expression of $\alpha_4\beta_7$ integrin is thought to be a characteristic of gut homing cells (Kuklin et al., 2000). While expression of these combinations of molecules partially correlates with the migration patterns of the cells, there does

appear to be a level of promiscuity in the use of certain homing molecules. For example, a population of $CD4^+$ T cells have been shown to utilise P-selectin and P-selectin glycoprotein ligand to migrate to the intestine (Haddad et al., 2003).

CCR7 has been shown to divide human memory T lymphocytes into two distinct subsets: $CCR7^-$ memory cells which express receptors for migration into inflamed tissues and show immediate effector function, while $CCR7^+$ memory cells express lymph node homing molecules and lack immediate effector function. These cells were termed effector (T_{EM}) and central memory (T_{CM}) (Sallusto et al., 1999) and have more recently been described in mice (Bjorkdahl et al., 2003). A recent study, in a murine model, carried out an investigation in the trafficking of effector memory $CD8^+$ cells in lymphoid and non-lymphoid tissues following viral or bacterial infection (Masopust et al., 2001). It was shown that antigen specific memory cells preferentially migrated to non-lymphoid tissues and that this migration was independent of the infectious agent used and the route used to administer the pathogen. A complementary study by the same group investigated the migration patterns of activated primary and memory $CD8^+$ cells. It was shown that activated $CD8^+$ T lymphocytes migrated extensively to all non-lymphoid tissues irrespective of the site of the initial antigen encounter. Also, using an adoptive transfer system, migration of these non-lymphoid populations was demonstrated to be promiscuous with a preference for homing to the tissue of origin. These results suggest that subsets of memory cells exist in each tissue and exhibit a tendency towards homing to their original site of activation (Masopust et al., 2004).

1.5.4.1 Chemokines and Tissue Specific Homing

Chemokines have been shown to be important in the role of cell arrest in the multistep model of lymphocyte homing (Campbell et al., 1998b). Lymphocyte expression of adhesion molecule and chemokine receptors directs their migration from the blood into tissues. Chemokines function at several steps in these homing pathways mediating firm arrest of lymphocytes rolling along vascular endothelium, promoting diapedesis, and segregating lymphocytes within tissues into functional microenvironments. The best characterised example of these chemokine functions are CCL21 and CCL19 which are involved in homing to secondary lymphoid tissues and mediate entry of naïve lymphocytes before segregation into distinct T and B cells areas (Muller et al., 2002).

Other chemokines trigger only certain specialised subpopulations. An example is CCL20 which triggers adhesion of CD4+ memory T cells but has no effect on naïve T cells (Campbell et al., 1998b). Another example is CCL17 which is expressed on skin endothelium, and functions in conjunction with CCR4 expression on skin-homing lymphocytes. CCL17 helps to mediate skin specific homing (Campbell et al., 1998a). This finding has brought about the idea that triggering of adhesion of cells to the endothelial layer may serve as a checkpoint, allowing only certain subsets of cells to enter a particular tissue.

Due to its restricted expression in the intestine, CCL25 has attracted considerable attention as a potential intestinal-specific homing chemokine (Kunkel et al., 2000, Uehara et al., 2002). Neutralisation of CCL25 in vivo with a blocking antibody, has directly shown that CCL25 and its receptor CCR9 function in the recruitment of effector lymphocytes to the small intestinal

epithelium following their activation in the GALT (Svensson et al., 2002). CCL25 has also been shown to play an essential role in the intestinal homing of IgA antibody secreting cells by mediating their extravasation into intestinal lamina propria (Hieshima et al., 2004). A closely related chemokine CCL28 has also been implicated in mucosal tissue-specific homing due to its expression by epithelial cells in several mucosal tissues including the trachea, lung, colon, mesenteric lymph nodes, salivary gland, and mammary gland (Pan et al., 2000, Wang et al., 2000).

1.5.4.2 Homing and Adhesion in the Respiratory Compartment

There has been little research carried out to investigate respiratory lymphocytes with regard to their trafficking patterns and homing receptors. Studies in animal models suggest that there is a distinct pattern of recirculation for respiratory tract T lymphocytes. In the mouse NALT, T cell binding has been shown to be L-selectin/PNAd mediated and not as a result of $\alpha_4\beta_7$/MAdCAM-1interactions, suggesting a closer link of these cells to peripheral lymph than to gut lymph (Csencsits et al., 1999). By contrast, in sheep, lung lymphocytes appear to have their own distinct migration profile due to their low expression of both L-selectin and of the $\alpha_4\beta_7$ integrin (Abitorabi et al., 1996).

More recently in a study focused on the homing of lymphocytes to the bronchial associated lymphoid tissue (BALT), it was noted that homing to this area is mediated by collaboration between L-selectin/PNAd, $\alpha_4\beta_1$/VCAM-1 and LFA-1/ICAM interactions. This is the first report of unique high level of $\alpha_4\beta_1$/VCAM-1 interaction in a secondary lymphoid tissue (Xu et al., 2003). This study also showed that anti-α4 and anti-VCAM-1 monoclonal antibodies were able to inhibit around 40% of total lymphocyte homing to the BALT, but more specifically

blocked 70% of the homing of a memory T cell rich T lymphocyte pool suggesting that $\alpha_4\beta_1$ and VCAM-1 are involved in the recruitment of memory T cells to the BALT.

The mechanism of lymphocyte homing to the lungs is not fully understood. In a model of murine pulmonary inflammation, ICAM-1 has been implicated in lymphocyte recruitment to the lungs (Chin et al., 1998). L-selectin and ICAM-1 have also been shown to play an important role in the migration of lymphocytes to the lungs in the murine asthma model (Keramidaris et al., 2001). Bacterial or viral infection has been shown to generate effector and memory $CD8^+$ cells that migrate preferentially to the non-lymphoid tissues including the lungs, suggesting these cells either continuously circulate through peripheral tissues or reside in them permanently (Masopust et al., 2001). However, there have been no reports specifically aimed at investigating the trafficking of cytotoxic $CD8^+$ T cells into the lungs. One recent study did show, by utilising monoclonal antibody blocking, that LFA-1 is essential in the retention of activated $CD8^+$ T lymphocytes in the murine lung (Thatte et al., 2003).

In the murine influenza virus model, it has been demonstrated that the integrin $\alpha_1\beta_1$ (VLA-1) is responsible for retaining protective memory $CD8^+$ T lymphocytes in the lung via attachment to the extracellular matrix. However recruitment is not VLA-1 dependent (Ray et al., 2004). It was observed that lung VLA-1 expressing $CD8^+$ lymphocytes were resistant to the apoptosis that follows recovery from influenza virus infection, and it is proposed that *in vivo* this resistance is conveyed through VLA-1 binding to its ligand type I or type IV collagen. It would appear that this resistance may allow the establishment and maintenance of a resident protective memory population and this finding is supported by the Masopust *et al.* (2001) study that demonstrated evidence of resident memory

cells and the preferential relocation of memory cells induced back into non-lymphoid tissues including the lung.

1.6.1 Aims of this investigation

Whilst the current influenza vaccines provide protection and are associated with an excellent safety record and are well tolerated (Beyer et al., 2002) there remains room for improvement. The influenza virus is continuously undergoing antigenic change driven by pressures to evade the acquired immunity of the population. Any antigenic shift exhibited by the circulating virus strains puts pressure on the manufacture of protective vaccine stocks. The circulating virus strains have to be constantly monitored so that accurate predictions can be made as to what vaccine strains are required. The vaccine must aim to protect against any new virus strains which appear virtually on an annual basis. The real threat is that of a pandemic, where highly pathogenic influenza virus strains pass from animal reservoirs to humans and cause fatal disease. If the next influenza pandemic was to strike tomorrow, inactivated vaccines could offer immediate prophylaxis, however their supply is limited and the production of more vaccine is time consuming and obviously dependent on a supply of embryonated hens eggs. On an annual basis, it is estimated that 6 months is required to organise sufficient eggs for vaccine manufacture (Gerdil, 2003). Taking this into consideration, vaccines based on conserved viral proteins are ideal candidates for a new influenza vaccine as they are highly conserved between different strains of virus and one vaccine should be efficient at controlling most influenza virus strains.

Investigations have been carried out using the internal conserved NP and M proteins and vaccinations with these have elicited protective immune responses

in mice. Some of these data were based on the protection generated following DNA immunisation which has been reported by several groups. This included an investigation into PMID of DNA as a method of immunisation. Ballistic gene gun immunisation has been shown to generate strong humoral and cellular responses but the analysis of this response has often been limited to the systemic immune system. As influenza virus infects via a mucosal route, it is of interest to assess the ability of PMID immunisation of DNA to elicit a mucosal immune response. The work presented was aimed at characterising the immune responses elicited by DNA immunisation administered to naïve mice by the PMID route. These results would be compared to the protective immune responses generated in a murine model of influenza virus infection. It is hoped that this detailed characterisation of both viral and vaccine induced immune responses will provide an insight as to whether PMID delivery of DNA can induce immune responses that may be required by a new generation of vaccines.

This study will initially characterise the immune response generated by influenza virus infection with regard to humoral and cellular immune responses. With this information, studies will continue analysing the immune response generated by PMID delivery of a plasmid coding for influenza virus NP. Comparison of these data should indicate desirable characteristics required of an influenza vaccine and assess PMID as a vaccine delivery method for a respiratory pathogen. Finally studies will characterise the antiviral cellular response induced by both infection and vaccination with regard to the homing characteristics of effector $CD8^+$ T cells. This analysis should assess how good PMID of NP DNA is at generating a response similar to that of experimental infection and predict its effectiveness as a vaccine.

1.6.2 Hypothesis to be Tested

It is hypothesised that virus-specific B and T lymphocytes will traffic extensively throughout the body by virtue of the CMIS following activation by either intranasal influenza virus infection or PMID of influenza virus NP DNA, regardless of their initial site of activation. This analysis will determine the efficacy of PMID of NP DNA at stimulating a broad anti-influenza immune response in terms of the strength and distribution of virus-specific cells when compared with influenza virus infection which normally produces sterilising immunity. The IEF technique was developed, in conjunction with fragment cultures, to aid in the identification of the production of anti-influenza antibodies by ASCs in various compartments. Influenza virus-specific cytokine ELISPOTs and flow cytometry analysis of tetramer positive anti-viral cells was developed to allow identification of the location of virus specific $CD8^+$ T cells following infection and immunisation and to elucidate their phenotype and homing patterns. Together these analyses will aid in the determination of the clonal distribution of virus-specific ASCs and whether virus-specific T cells identified in different compartments of the immune system have similar homing properties regardless of their method of activation.

Chapter 2

Materials and Methods

2.1 Mice

Adult, 6-8 week old, female C57BL/6 mice (H-2^b) were obtained from Charles River Laboratories UK and were maintained in specific pathogen free conditions at the Institute for Animal Health, Compton, UK or at GSK, Stevenage, UK. The mice were allowed to acclimatise for at least seven days before the start of a study. The animals were housed in groups of up to 10 and checked daily throughout each experiment.

2.2 Virus

Influenza virus A/PR/8/34 (H1N1) was purchased from Charles River SPAFAS (North Franklin, CT). This was provided as a preparation of allantoic fluid obtained from specific pathogen free chicken embryos infected with influenza virus A/PR/8/34. The influenza virus was used to infect mice intranasally with a sub-lethal concentration of 500 EID_{50} (50% egg infectious doses) in 30μl of PBS.

2.3 Isolation of Immune Cells

At certain time points post infection, mice were sacrificed for collection of various tissues of the mucosal and systemic immune system. Briefly, the following tissues were collected from each animal and placed in Hanks' balanced salt solution (HBSS; GIBCO Biomedical research laboratories, Grand Island, N.Y.): spleen, lungs, gut, hind legs (for bone marrow), mediastinal lymph node (MedLN), mesenteric lymph nodes (MesLN) and head (for NALT, which was not placed in HBSS). Each tissue was treated slightly differently to prepare it for incubation as fragment cultures (to investigate secreted immunoglobulins) or to isolate the lymphocytes, as described below:

Lymph nodes

Fat was dissected away from the lymph nodes. Prior to incubation as fragment cultures each lymph node was sliced in half, and both pieces were placed in 1ml of RPMI 1640 medium (GIBCO) supplemented with 10% foetal calf serum, penicillin and streptomycin to final concentrations of 100IU and 100μg/ml respectively, and amphotericin at a final concentration of 0.25μg/ml (hereafter referred to as RPMI). Cells were isolated from lymph nodes for immune assays by placing in 5ml RPMI and rubbing each node between the frosted ends of sterile glass slides to disaggregate the cells. The resulting cell suspension was harvested by centrifugation. Cell suspensions were washed twice using PBS and the cells were counted (using Trypan Blue exclusion and a haemocytometer or using an automated Guava counter).

Spleen and lung

Spleen and lung tissues were cut into 1-2mm fragments and placed in 1ml of RPMI medium prior to fragment culture. Cells for immune assays were isolated by placing in 10ml RPMI and rubbing spleen or lung tissue between the frosted ends of sterile glass slides to disaggregate the cells. The resulting cell suspensions were harvested by centrifugation and any contaminating erythrocytes were lysed by incubating the samples for 1 minute with red blood cell lysing buffer (Sigma, UK). Cell suspensions were washed twice using PBS and the cells were counted (using Trypan Blue exclusion and a haemocytometer or using an automated Guava counter).

Bone marrow

Tissue was dissected away from the hind legs to expose the femur which was divided into 3 sections, each of which was placed in 1ml of RPMI. Bone marrow was only used in fragment culture analysis.

O-NALT

The O-NALT was isolated by the method described Asanuma et al (1997). Briefly, the head of the mouse was collected and fur removed from the entire head. The eyes and loose tissues behind the eyes were removed before the lower jaw was removed to expose the palate. Using a scalpel, a transverse cut was made behind the eye sockets and the back portion of the head and remnants of brain tissue were discarded. The tip of the nose containing the foreteeth was removed, as were the cheekbones and the cheek muscles. The O-NALT was identified and isolated under a dissection microscope. It was visualised as two small longitudinal lymphoid strips located on the posterior side of the palate. The whole palate was removed under the dissection microscope and the O-NALT was peeled off the palate using fine forceps. Cell suspensions were made by rubbing the O-NALT between the frosted ends of sterile glass slides to disaggregate the cells.

D-NALT

Heads were prepared as for the O-NALT and after removing the palate, the teeth were cut away. The remaining nose part consists of the nasal bones, nasal turbinates and the septum. This nose part was cut into very small pieces and transferred into DMEM/10%FCS containing 4mg/ml collagenase A (Roche Molecular Biochemicals, UK). This suspension was incubated with gentle rocking at 37°C for 30 minutes. The digested mixture was strained through a 70μm cell strainer and centrifuged at 1400rpm for 5 minutes. It was then resuspended in 5 ml DMEM/10% FCS.

A Percoll (Amersham Pharmacia, UK) density gradient was prepared by layering 2ml 40% Percoll over 2ml 75% Percoll. The 5ml D-NALT suspension was carefully layered onto the gradient before centrifugation at 2000rpm for 20 minutes at room temperature (RT). D-NALT lymphocytes were removed from the interface of the 40% and 75% layers with a sterile glass Pasteur pipette and transferred into a new 15ml

Falcon tube. Isolated cells were washed with DMEM/10% FCS. The pellet was resuspended in 1ml DMEM/10% FCS. For incubation as fragment cultures the D-NALT was left intact and cut into three fragments, each of which was placed in 1 ml RPMI.

Peyer's patches and Gut

Peyer's patches were removed from the gut, cut in half and placed in 1ml RPMI. Sections of approximately 5 cm from the duodenum, ileum and colon of the gut were cut longitudinally to expose the mucosal surface, before several 2mm sections were collected. The gut sections were washed 6 times in PBS containing 0.05% EDTA, 0.25μg/ml amphotericin, penicillin and streptomycin at final concentrations of 100IU and 100μg/ml respectively before being placed in 1ml of RPMI for incubation as fragment cultures. Only cells from the Peyer's patches were isolated for immune assays by placing them in 10ml RPMI and rubbing each Peyer's patch between the frosted ends of sterile glass slides to disaggregate the cells.

Serum

When animals were Schedule 1 culled whole blood was collected in to Serum Separation tubes (BD Biosciences, San Jose, CA). Serum was separated by centrifugation at 14000 rpm for 3 minutes. Serum was stored at -20°C until analysis.

2.4 Fragment Culture Method

Cultures of tissues were prepared by an adapted technique previously described for culture of small intestinal lymphoid tissue (Khoury et al., 1994). All the fragment cultures, prepared as described above, were incubated at 37°C in a humidified environment of 95% oxygen 5% carbon dioxide for 5 days after which the fragment culture medium was removed and stored at -20°C for analysis.

2.5 Isoelectric Focusing Using the BioRad Criterion™ System

A Criterion IEF gel pH3-10 (BioRad Laboratories, Hercules, CA) was prepared and inserted into the Criterion tank. The upper buffer chamber was filled with 1× Cathode buffer (BioRad) and the reservoir of the tank was filled with 1× anode buffer (BioRad). Samples were mixed in a 1:1 ratio with IEF sample loading buffer (BioRad) before loading into the gel along with 5 µl of undiluted IEF standards pI 4.45-9.6 (BioRad). The samples were separated by electrophoresis on the pH 3-10 gradient gel under the following 3 step protocol: 100V for 1 hour, 250V for 1 hour and 500V for 30min.

Various protein visualisation techniques were investigated which will not be discussed here since protein staining following IEF separation using the PhastSystem proved to be far superior.

2.6 IEF Using the Amersham Biosciences PhastSystem™

Samples were separated by electrophoresis on a PhastGel™ IEF 3-10 (Amersham Biosciences, Uppsala, Sweden). Briefly, the ampholytes within the gel were allowed to prefocus at 2000V for 75Vh to create a pH gradient, followed by sample application at 200V for 15Vh, and focusing at 2000V for 410Vh.

Proteins were visualised using Plus One Coomassie Tablets, PhastGel™ Blue R-350 (Amersham Biosciences) and PhastGel™ Protein Silver Staining Kit (Amersham Biosciences) and the results were compared to those obtained using the BioRad Criterion system.

2.7 Protein Transfer and Immunoglobulin Visualisation

Two techniques were established to visualise the virus specific immunoglobulin proteins once they had been transferred to HYBOND-P PVDF membrane (Amersham Biosciences) as follows:

Active Transfer

Towbin transfer buffer was prepared and chilled to 4°C to improve heat dissipation. The buffer comprised 25mM Tris, 192mM glycine, 20% (v/v) methanol, pH8.3. After electrophoresis, the gel was equilibrated in transfer buffer for 15min, before a gel/PVDF membrane sandwich was created in the transfer cassette of the Criterion Blotter™ (BioRad) as described in the manufacturer's protocol. Transfer of protein was carried out under electrophoresis at 100V for 60min.

Following transfer of protein, unbound areas of the membrane were blocked for 1 hour at RT by incubating in a 5% (w/v) solution of Marvel (powdered milk) in 1× Tris–buffered saline (TBS) (10× comprising 20mM Tris, 500mM sodium chloride, pH 7.4). The membrane was incubated at 4°C, under agitation, with a 0.2mg/ml solution of sucrose purified Influenza A/PR/8/34 (Charles River SPAFAS) in TBS/0.5% (w/v) Marvel for 4 hours. The membrane was washed 4 times in TBS/0.05% (v/v) Tween-20 for at least 5 min/wash. The membrane was treated with a 3% (v/v) hydrogen peroxide solution in TBS/0.05% (v/v) Tween-20, at room temperature for 2 min under agitation. The membrane was washed 4 times as before, followed by incubation with goat anti-influenza A (H1N1)-HRP (BioDesign International, Saco, Maine) at a dilution of 1 in 5000 made up in TBS/0.5% (w/v) Marvel/0.05% (v/v) Tween-20. The membrane was washed 4 times as before, prior to visualising the protein using an ECL Western blotting detection system (Amersham Biosciences) using the method described in the manufacturer's protocol.

The influenza specific antibodies were also visualised using incubation with goat anti influenza A-biotin (BioDesign International) optimised to a dilution of 1 in 5000. This was followed by a wash step, as before, prior to incubation with streptavidin-HRP (DAKO, Denmark) at a dilution of 1 in 20 000 in TBS/0.05% (v/v) Tween-20. The proteins were visualised using chemiluminescence as described above.

Passive Transfer

Virus specific immunoglobulins were transferred from the gels by passive diffusion onto a membrane precoated with a 10μg/ml solution of sucrose purified Influenza A/PR/8/34 (Charles River SPAFAS) in TBS/0.5% (w/v) Marvel. Membranes were coated overnight with agitation at 4°C, before blotting was carried out. Following transfer, unbound areas of the membrane were blocked for 1 hour at RT using a 5% (w/v) solution of Marvel in 1× Tris–buffered saline (TBS) (10× comprising 20mM Tris, 500mM sodium chloride, pH 7.4). The membrane was incubated at RT for 1 hour, with agitation, in a 1:1000 dilution of goat anti-mouse Ig (H+L)–HRP (Southern Biotechnology, Birmingham, Alabama), in 1×TBS/1% (w/v) Marvel/0.1% (v/v) Tween 20. The membrane was washed 4 times, before immunoglobulin was visualised using an ECL Western blotting detection system (Amersham Biosciences).

2.8 Analysis of Influenza A/PR/8/34 Monoclonal Antibodies

The following monoclonal antibodies (MAbs), with specificity for Influenza A/PR/8/34 haemagglutinin (HA), were kindly supplied as hybridomas by Prof. Nigel Dimmock (University of Warwick): H36-4.5-2 (IgG2a), H37-45-5R3 (IgG3), H37-66-1 (IgA), H9-D3-4R2 (IgG3). MAb supernatants were prepared as previously described (Yokoyama, 2000).

The MAbs were analysed by IEF (BioRad Criterion™ system and Amersham PhastSystem™) and Influenza A/PR/8/34 specific immunoblotting, as previously described.

2.9 Fragment Culture Concentration

Selected fragment cultures were concentrated by centrifugation using Vivaspin 6 columns-10^6 MWCO (VivaScience, Sartorius Group, Goettongen, Germany) to investigate the effect of concentrating the proteins before separating them by IEF. The columns feature twin vertical membranes and a dead space to prevent drying out of samples and allow collection of concentrated sample.

2.10 Quantitation of Anti-Influenza Antibodies by ELISA

In order to quantify the amount of anti-influenza antibodies, three ELISA assays were developed. The first, a capture ELISA was used to create an isotype specific standard curve, the second replaced Ig standards with fragment cultures to directly quantify the amount of each Ig isotype present in a fragment culture. The third ELISA was virus-specific and was used to indirectly quantify the anti-influenza antibodies.

Capture ELISA

The wells of Nunc-Immuno 96 well plates (Nalge Nunc, Rochester, NY.) were coated overnight at 4°C with 100µl of unlabelled anti-Ig isotypes (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM), at a concentration of 5µg/ml in coating buffer (comprising 20mM Tris HCl pH 8.5). The plate was washed three times in Phosphate buffered saline (PBS) before blocking any unbound regions with a solution of PBS/1% (w/v) Marvel/0.05% (v/v) Tween-20 for 1 hour at room temperature. Purified mouse Ig standards IgG1, IgG2a, IgG2b, IgG3, IgA, IgM (Sigma, Poole, England) were diluted to a starting

concentration of 10μg/ml before creating serial 2-fold dilutions. One hundred microlitre volumes were used to coat the corresponding anti-isotype wells for 2 hours at room temperature. The wells were washed as before, with a subsequent incubation with 100μl of anti-isotype AP conjugated antibodies at RT for 2 hours. One final wash step was carried out as before. The development reagent was prepared by dissolving one p-nitrophenyl phosphate tablet (Sigma, UK) in 15ml diethanolamine buffer. One hundred microlitre volumes of development reagent were added to each well after the wash step and development was allowed to occur until a colour change was observed. At this point, the reaction was stopped by the addition of 50μl of 3M NaOH to each well and the optical densities were measured at 405nm.

Capture ELISA for analysis of fragment culture samples

The protocol used was identical to that described above however, the Ig isotype standards were replaced with fragment culture samples.

Influenza Virus Specific ELISA

Sucrose purified Influenza A/PR/8/34 2mg/ml (Charles River SPAFAS) was detergent disrupted in a 10 % (v/v) detergent buffer solution (10× comprising, 0.05M Tris pH7.5, 0.5% Triton-X-100 and 0.6M KCl.) and subsequently diluted to a concentration of 5μg/ml. One hundred microlitre volumes of the detergent disrupted influenza virus were used to coat wells of Nunc immuno plates overnight at 4°C. Any unbound regions were blocked by incubating 200μl of a solution of PBS/1% (w/v) Marvel/0.05% (v/v) Tween-20 in each well for 1 hour at RT. The wells were washed as described above. Serial dilutions of fragment culture were prepared and 100μl volumes were incubated in appropriate wells for 4 hours at RT. The wells were washed as previously described before development with alkaline phosphatase conjugated antibodies as described above.

2.11 IFNγ ELISPOT – Influenza NP Peptide Stimulation

Wells of a 96 well Multiscreen-IP plate (Millipore, Watford, UK) were coated with 50μl of rat anti-mouse IFNγ (BD Pharmingen, San Diego, CA) diluted to a concentration of 15μg/ml in PBS. Plates were incubated overnight at 4°C, or at RT for at least 4 hours. Plates were washed twice with PBS and blocked using 200μl RPMI for 2 hours at 37°C.

Cells were collected, isolated, washed, counted and diluted in RPMI to a final concentration of either 2 or 4×10^6 cells/ml. Murine recombinant IL-2 was added to the cells at a concentration of 10U/ml and 100μl aliquots of cell suspension were plated out in triplicate. $NP_{366-374}$ peptide (ASNENMETM) was diluted to a concentration of 20μg/ml in RPMI, and 100μl volumes were mixed to each well (giving a final concentration of 10μg/ml). Plates were incubated for 16-30 hours at 37°C. (16 hours was found to be sufficient for strongly responding cells e.g. lungs and MedLN but other tissues e.g. NALT and PP required a longer incubation of 30 hours).

Plates were washed 4 times with PBS before incubation with 50μl anti-IFNγ-biotin (BD Pharmingen) at a 1:1000 dilution in PBS for 2 hours at RT with shaking (or incubated overnight at 4°C). The plates were washed four times with PBS before incubation with 50μl goat-anti-biotin-AP (Vector Labs, UK) at a 1:1000 dilution in PBS for 2 hours at RT with shaking (or overnight at 4°C).

Plates were washed a final time before development with 50μl of Bio-Rad AP conjugate substrate prepared according to the kit manufacturer's protocol. Plates were left to develop in the dark at RT and, when purple spots appeared (about 10 mins) the reaction was stopped using three washes of distilled and deionised water.

2.12 Tetramer Staining

Single cell suspensions from pooled lymphoid tissues of individual mice were prepared and influenza specific cells were analysed using phycoerythrin (PE) conjugated H-2Db $NP_{366-374}$ tetramer (ProImmune, Oxford, UK) displaying the dominant NP CD8 epitope ASNENMETM. Before tetramer staining, cell suspensions were enriched for $CD8^+$ T cells. Briefly, cells were incubated with a 1:100 dilution of the following monoclonal antibodies: TIB 120 (anti-MHC II), RA3-6B2 (anti-B220) and GK1.5 (anti-CD4) for 1 hour with rotation at 4°C. Cells were collected by centrifugation before incubation with 1×10^7 Dynabeads/ml (Dynal Biotech, Norway) for 30min with rotation at 4°C. Cells with bound monoclonal antibody and Dynabeads were removed using a magnet and the remaining cells were washed in DMEM medium supplemented with 10% FCS.

For the tetramer staining, 1-2 × 10^6 cells of a suspension were washed in PBS/0.1% BSA before being incubated with one test of the tetramer (2μl) for one hour in the dark at room temperature. The cells were washed and counterstained with fluorescein isothiocyanate (FITC) conjugated anti- CD8 (BD Pharmingen, San Diego, CA) for 30 min in the dark at 4°C. Cells were washed one final time, before resuspension in 300μl of the wash buffer and detection of immunofluorescence on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

The first tetramer staining experiment also investigated indirect tetramer staining following stimulation of cell suspensions with irradiated influenza virus infected splenocytes. This was carried out to stimulate lower frequency populations to a frequency detectable by FACS analysis. Briefly, lymphocytes were prepared from 2 naïve spleens before being incubated with 1ml of influenza virus A/PR/8/34 infected allantoic fluid in a 37°C water bath for 2 hours. The cell/virus suspension was made

up to 10ml with DMEM/10% FCS and incubated for a further 2 hours in a 37°C water bath. Cells were irradiated at 3000rads before being diluted to 2×10^6 cells/ml. Equal numbers of irradiated stimulator cells were incubated with cell suspensions from the various tissues at 37°C for 7 days. Cells were then harvested by centrifugation and stained with tetramer and analysed as before.

2.13 Virus Specific Lymphocyte Phenotyping

Single cell suspensions were prepared from pooled lymph nodes (inguinal, mediastinal, mesenteric), spleen, Peyer's patches, lungs and D-NALT (prepared as previously described) in PBS/0.1% BSA. Cells were initially enriched for $CD8^+$ T cells (as previously described) before being stained with tetramer (as previously described) and counterstained with various antibodies for 30 min in the dark at 4°C. Antibodies used were as follows: FITC conjugated anti-CD44, anti-CD11a, anti-CD62L, anti-CD69 and Cychrome-5 (Cy5) conjugated CD8. PE conjugated $\alpha_4\beta_7$ integrin was used in conjunction with, FITC conjugated anti-CD8 and allophycocyanin (APC) conjugated pentamer (ProImmune, Oxford, UK). The pentamer staining protocol was the same as for tetramer staining, however one test used 10μl of the reagent. All antibodies were obtained from BD Pharmingen (San Diego, CA) except anti-CD8-Cy5 which was conjugated on site and a gift from Dr Agnes Le Bon (Edward Jenner Institute). Detection of immunofluorescence was carried out on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

2.14 PMID Cartridge Preparation

The following details and equations were required in the preparation of DNA cartridges:

- Ethanol used must be dried and stored for at least 1 week in a desiccator.
- Roughly 35mg of gold (2μg particle size, Powderject/Chiron, Madison, WI) is required to make 40 cartridges
- The DNA loading rate (DLR) is 2, which results in approximately 0.5 µg DNA/cartridge. Amount of DNA required = (weight of gold (µg) x DLR) / concentration of DNA (µg/ml)
- 0.05M spermidine (Sigma, UK) is pre-aliquoted and stored at -20°C. The amount of spermidine required relates to the volume of plasmid to be used. If volume of plasmid < 100µl, amount of spermidine = 100µl. If the volume of plasmid is > 100µl, the same volume of spermidine as volume of plasmid is used.
- Volume of ethanol PVP required = amount of gold (µg) / 8.75 which is the amount of gold required per ml of ethanol to give roughly 0.5µg gold per cartridge.

The concentration of plasmid DNA present was quantified using GeneQuant (Biochrom, UK). This was required to calculate the amount of DNA required for the number of cartridges required (which was calculated using the equation shown above). Gold and spermidine were sonicated together for 30 seconds, and mixed on a vortex mixer prior to addition of the DNA. A volume of calcium chloride, equal to that of the spermidine, was added before immediate mixing on a vortex mixer. The DNA was allowed to precipitate onto the gold for 10 min. Meanwhile, a 10mg/ml fresh stock of PVP (polyvinyl pyrrolidone, Sigma, UK) was prepared in dried ethanol before subsequent dilution to a working concentration of 0.15µg/ml in ethanol. The gold was

centrifuged for a few seconds at 14000 rpm. The gold/plasmid pellet was washed three times with 1ml of dried ethanol, resuspending the pellet between washes.

After the third wash, the ethanol was removed and the pellet resuspended. The calculated volume of ethanol/PVP was placed in a glass vial before 180µl of the ethanol/PVP mix was used to transfer some of the plasmid coated-gold. This step was repeated until all of the gold had been transferred into the ethanol/PVP mix.

The ethanol/PVP/gold mix was loaded into the tube turner which was already preloaded with tefzel tubing (Powderject/Chiron, Madison, WI). A flow of humidified nitrogen through the spinning tubing was used to ensure even coating of the DNA/gold. The spin rate helped to expel the excess ethanol and an increased nitrogen flow assisted with drying the plasmid/gold coated tubing. The tubing was cut into even 1.27cm lengths (cartridges) using a BioRad tubing cutter. The cartridges were stored with a silica gel capsule at RT overnight to allow the silica gel to dehydrate the cartridges further, before longer term storage at 4°C. Cartridges were freshly prepared for each experiment.

For every batch prepared the actual amount of DNA coated onto the cartridges was determined by eluting the DNA from two cartridges. A 50µl volume of DNase/RNase free water was carefully added to the centre of each of two cartridges in a 1.5ml Eppendorf tube, before incubation for 30 min at 37°C. Following incubation, the water/gold was spun out of the cartridges by centrifugation for a few seconds in a Microfuge at 14000 rpm. The eluent was pooled and quantified on the GeneQuant and using the following equation - *µg DNA / cartridge = GeneQuant reading (µg/ml) / 20*, and the amount of DNA in each cartridge was calculated.

Several cartridges from each plasmid batch were also test fired into Mylar (DuPont Teijin Films, UK) layered over parafilm on a perspex sheet. The penetration of the gold particles was assessed by densitometry of the parafilm to confirm a uniform firing pattern from each cartridge. Mylar is a polyester film which is strong and puncture resistant and the Mylar/parafilm combination was suggested by PowderJect/Chiron as a suitable skin substitute for testing cartridge firing/gold penetration for quality control purposes.

2.15 DNA Immunisation

The abdomen of each animal was shaved prior to immunisation with two cartridges by PMID at 500psi helium gas pressure using the BioRad Helios gene gun (BioRad Laboratories, Hercules, CA). In a typical vaccination, each cartridge contained 0.5mg gold coated with approximately 0.5µg plasmid DNA.

For transfer studies and phenotypic analysis, mice were immunised with two doses at day 0 and again at day 7. This had the effect of boosting the antigen specific cellular response (personal communication with Dr Fiona Cook).

2.16 Virus Titration

Specific pathogen free 10 day old embryonated hen eggs were provided by The Institute for Animal Health (Compton, Berks). These were used as a medium in which to propagate influenza infected lung homogenates using the method previously described (Mahy, 1985). Briefly, lung homogenates were prepared in 1ml PBS/gentamicin (50µg/ml) using a tissue homogeniser (PRO200 homogenizer with Multi-Gen 7 generators, PROscientific, Oxford, CT). Ten-fold serial dilutions of the homogenised tissue were made before 100µl of each dilution was used to infect the

allantoic cavity of the eggs in triplicate. The eggs were incubated at 37°C for 48 hours. After incubation, embryos were killed at 4°C overnight, before 50µl aliquots of the infected allantoic fluid was harvested from each egg and placed in the wells of a round bottomed 96 well plate. The presence of influenza virus was detected by utilising the ability of the virus to agglutinate red blood cells. Chicken red blood cells were washed 3 times in saline and diluted to a 1% working stock before the addition of 50µl to the aliquot of allantoic fluid. The red blood cells and allantoic fluid were left undisturbed for 45 min on a white surface. If no virus was present, the red blood cells formed a small pellet. A positive result was observed as a continuous sheet of agglutinated red blood cells.

The highest amount of infectious virus in a lung homogenate may be estimated by measuring the highest dilution that can still infect an embryonated egg. The smallest amount of virus capable of doing this, on 50% of occasions, is known as the egg infections dose (EID_{50}). Virus titre in EID_{50} was calculated as previously described (Mahy, 1985).

2.17 Transfer Experiments

The homing properties of naïve and effector $CD8^+$ T lymphocytes from C57BL/6 Ly5.1 mice were investigated in an adoptive transfer model. Effector $CD8^+$ cells were generated in the influenza model by intranasal infection with 500 EID_{50} influenza virus A/PR/8/34 or by PMID immunisation with two doses of 0.5μg influenza NP DNA as previously described.

Total lymphocytes were isolated from 20 spleens and enriched for $CD8^+$ T cells before $CD8^+$ cells were sorted using a MoFlo (DakoCytomation, Denmark). These cells had been labelled with 10 times the amount of antibody used for FACSCalibur

analysis. Briefly, spleens were pooled into groups of 5 and cell suspensions were prepared before staining with a 1 in 20 dilution of FITC conjugated anti-CD8 antibody for 30 min in the dark at room temperature. Cell suspensions were washed before being pooled into one final group and resuspended to 1×10^7 cells/ml. Following MoFlo isolation, $CD8^+$ T cells were washed 3 times in PBS before 1×10^7 cells were transferred intravenously in naïve C57BL/6 Ly5.2 recipients.

Two days following transfer, recipient animals were culled and lymphocytes were isolated from the spleen, liver, lungs, IngLN, MedLN, MesLN, NALT and Peyer's patches before they were stained with influenza specific tetramer (as previously described) and counterstained with FITC conjugated anti-Ly-5.1 and peridinine chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5) conjugated anti-CD8. All antibodies were obtained from BD Pharmingen (San Diego, CA) and immunofluorescence was detected using a FACSCalibur and CellQuest software (BD Biosciences, SanJose, CA.).

Chapter 3

Characterisation of Murine Humoral and Cellular Responses to Intranasal Infection with Influenza Virus A/PR/8/34

3.1 Introduction

Infection with live influenza virus can result in sterilizing immunity. Experiments were designed to characterise the immune cells and factors that contribute to this protective response. The aim of the proposed work was to quantitate the immune responses and mechanisms contributing to the *in vivo* induction of sterilising immunity generated using a murine model of influenza virus infection and to compare the immune responses with those elicited following vaccination.

A fragment culture technique was developed which allowed collection of specific antibodies produced by antibody forming cells (AFCs) isolated from various areas of the mucosal and systemic immune systems. ELISAs could then be used to measure how much virus-specific antibody was secreted from each tissue "fragment". ELISPOT assays were used to enumerate the virus-specific IFNγ secreting $CD8^+$ T cells in the various tissues which, according to the literature is a more sensitive method for detecting the presence of low level populations of antigen specific $CD8^+$ lymphocytes (Karlsson et al., 2003). An attempt was also made to measure the clonal populations of virus-specific antibody produced after infection in terms of size and location. Using isoelectric focusing gels, the separation of serum and fragment culture supernatants into their respective charges, based on amino acid structure, allows the analysis of clonal populations in terms of number of "bands". Determination of whether or not the same bands were found in different tissues could give an indication of the migration of clonal populations of AFCs.

Results gained from these initial studies will give an indication of what needs to be stimulated by a vaccine candidate in order for it to be successful not only in

terms of the strength of response but also in terms of the breadth and anatomical location of responding cells.

3.2 Results

3.2.1 Murine Anitbody Responses to Influenza Virus Infection are Dominated by Those Seen in the Mediastinal Lymph Node

At certain time points post infection, mice were sacrificed for collection of various tissues of the systemic and mucosal immune systems. Cultures of these tissues were prepared by a technique adapted from that previously described for culture of small intestinal lymphoid tissue (section 2.4).

In order to quantify the amount of specific anti-influenza virus antibodies produced by the fragment cultures, three modifications of enzyme-linked immunosorbent assays (ELISA) were developed. The first, a capture ELISA was used to create an immunoglobulin (Ig) isotype specific standard curve. The second replaced Ig standards with fragment culture supernatant to directly enumerate the quantities of each Ig isotype present in each fragment culture. The third ELISA was designed to indirectly quantify the anti-influenza virus antibodies present in fragment cultures.

The data generated from the analysis of fragment cultures showed a high sensitivity with a detection limit equivalent to 1ng/ml Ig. The immune response to influenza virus was observed to be dominated by cells and tissue fragments isolated from the MedLN. Immunoglobulin production by fragment cultures at various times post influenza virus infection is shown in Figure 4.

The results shown in Figure 4 demonstrate that influenza virus infection upregulates total antibody secretion (across all Ig isotypes) detected in the fragment culture supernatants. The graph for day 0 shows a background Ig production in most tissues, but predominantly in the gut. This result was expected considering the constant assault of this area by antigen. As the infection progresses all isotypes were seen to be upregulated with responses peaking around day 10, before the IgG levels diminish and IgA production increases. A dominant splenic IgM response is also observed.

The results of influenza virus-specific antibody production are shown in Figure 5. The initial virus-specific antibody response was observed in the MedLN, before being detected in the lungs and the D-NALT. IgG isotypes are the dominant isotype early in the virus-specific response however, as the infection is cleared a large increase in virus-specific IgA production is observed.

The proportion of virus-specific Ig produced is shown in Figure 6. Most of the IgA produced in the MedLN is 100% virus specific as the infection is cleared with lower levels of viral specificity (<25%) observed for the other Ig isotypes.

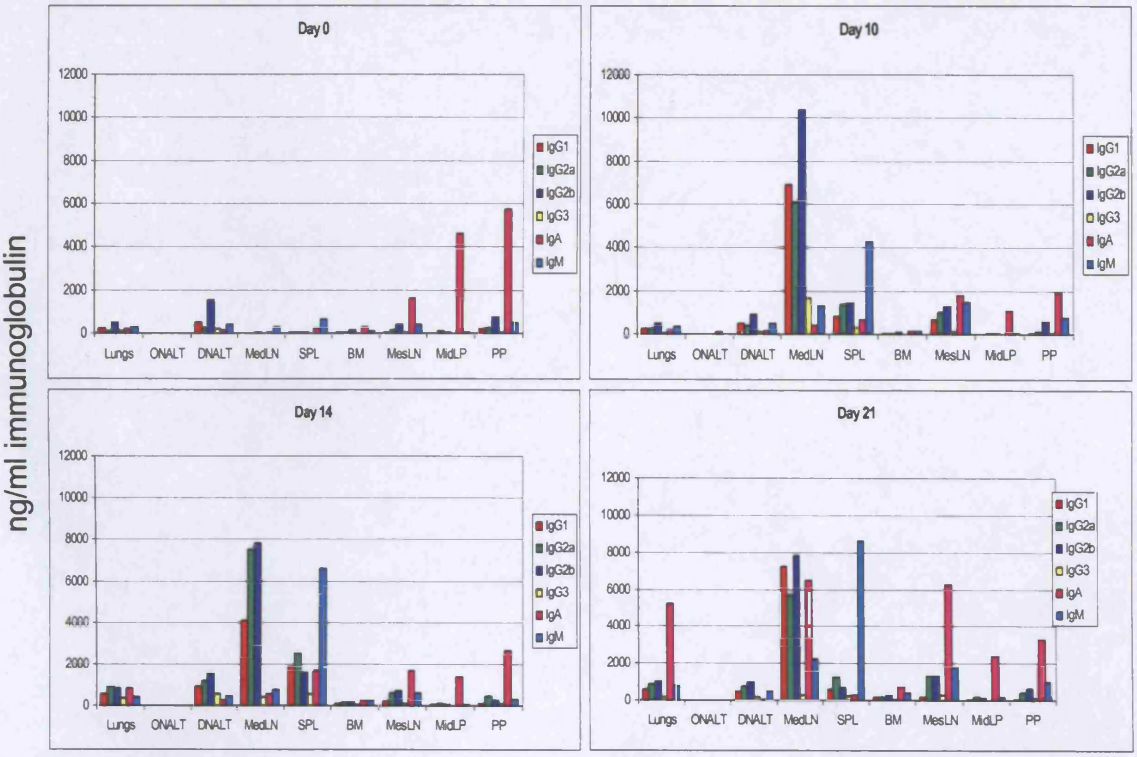


Figure 4: Total Ig production as calculated by ELISA is seen to increase in all tissues examined following intranasal influenza virus infection. 12 female C57BL/6 mice were intranasally infected with 500 EID_{50} influenza A/PR/8/34 virus while 3 C57BL/6 mice remained uninfected. Fragment cultures were prepared from various tissues of the mucosal and systemic immune system prior to (day0) and 10, 14 and 21 days post influenza virus infection. Fragment cultures from 3 individual mice per timepoint were pooled before the amount of total Ig within the samples was calculated. All tissues are seen to exhibit a dramatic increase in Ig production after influenza virus infection which is clearly dominated by the response observed in the MedLN. This data is generated from fragment cultures pooled from 2 experiments.

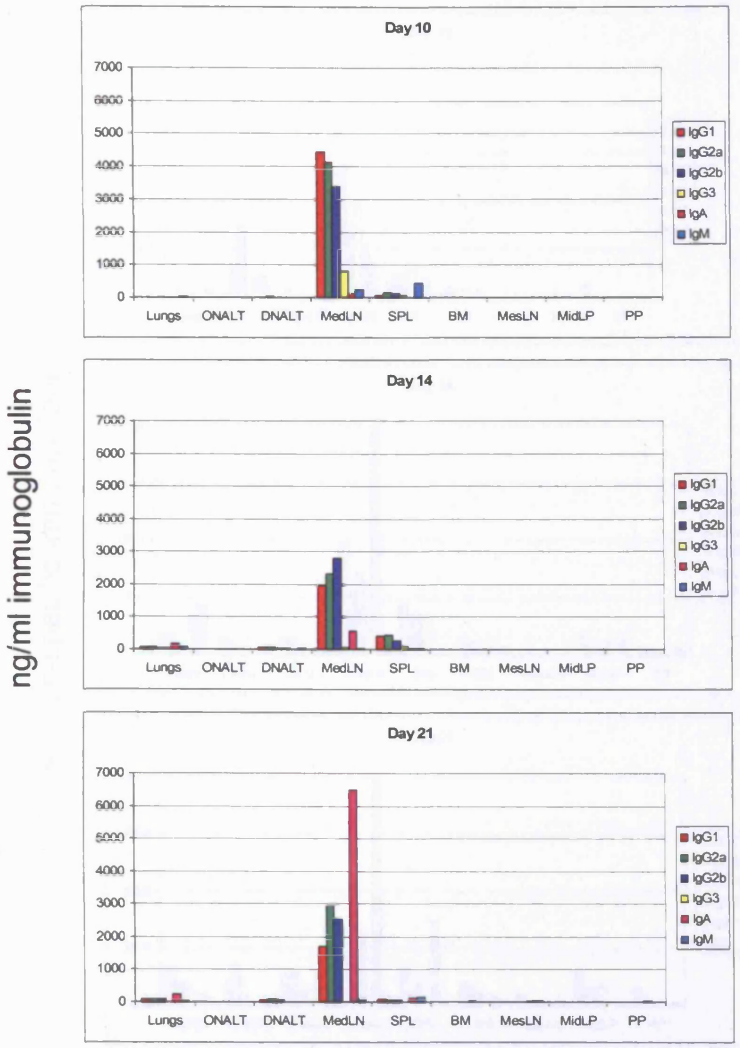


Figure 5: Virus-specific Ig is seen to be predominantly produced in the MedLN. 12 female C57BL/6 mice were intranasally infected with 500 EID_{50} influenza A/PR/8/34 virus and fragment cultures were prepared from various tissues of the mucosal and systemic immune systems. Fragment cultures from 3 individual mice per timepoint were pooled before the amount of virus-specific Ig within the samples was calculated at days 10, 14 and 21 following infection. The MedLN response dominated throughout the timecourse with spread to the respiratory compartment and spleen seen in later timepoints. Day 21 show a predominance for IgA class Ig in the MedLN. This data is generated from fragment cultures pooled from 2 experiments.

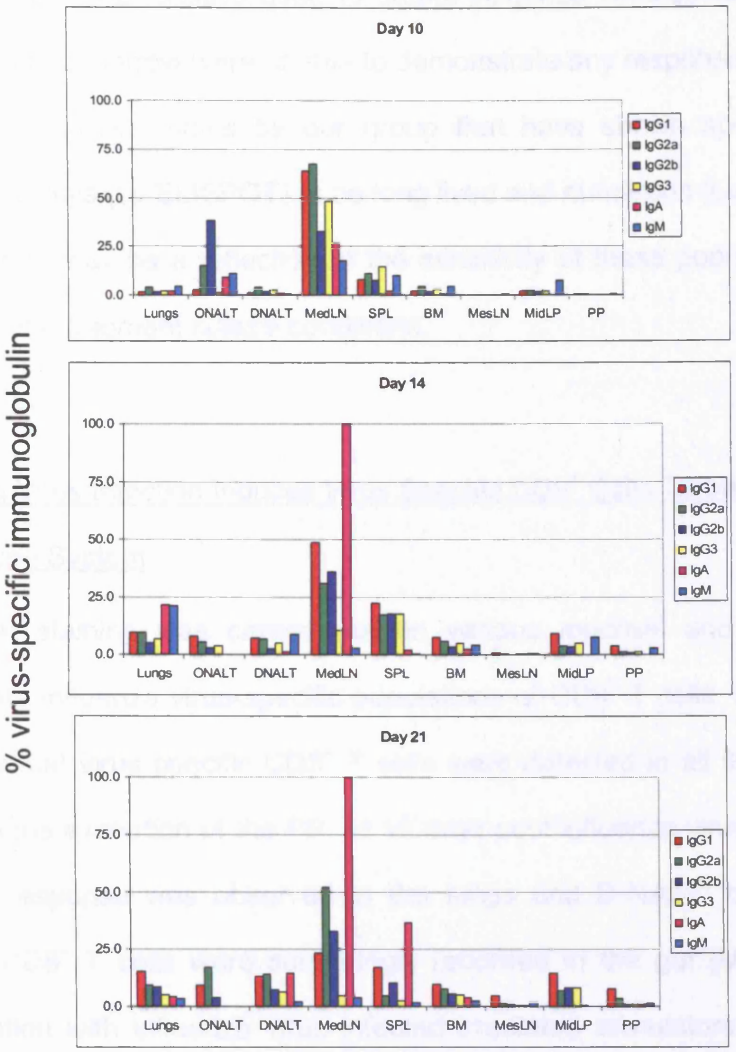


Figure 6: As influenza virus infection is cleared, 25-50% of the IgG isotypes and 100% of IgA produced in the MedLN is influenza virus-specific. 12 female C57BL/6 mice were intranasally infected with 500 EID_{50} influenza A/PR/8/34 virus and fragment cultures were prepared from various tissues of the mucosal and systemic immune systems. The relationship between total Ig (Figure 4) and influenza virus-specific Ig (Figure 5) was calculated, and shown as a percentage of virus-specific Ig in the total Ig pool. This data is generated from fragment cultures pooled from two experiments and the method of analysis was adapted from a method previously described by Khoury *et al.*, (1994).

Unfortunately, the ELISA assays used to detect influenza virus-specific IgA in the D-NALT or bone marrow were unable to demonstrate any responses. This is in contrast to previous studies by our group that have shown specific IgA antibody forming cells (by ELISPOT) to be long lived and numerous (Liang et al., 2001). This result may be a reflection of the sensitivity of these populations of plasma cells to the fragment culture conditions.

3.2.2 Influenza Virus Infection Induces Virus Specific $CD8^+$ Cells Throughout the Mucosal Immune System

Direct tetramer staining was carried out on various mucosal and systemic tissues to locate influenza virus-specific populations of $CD8^+$ T cells. Results in Figure 7 show that virus specific $CD8^+$ T cells were detected in all the tissues examined with the exception of the PP. At 10 days post influenza virus infection the dominant response was observed in the lungs and D-NALT. In addition virus-specific $CD8^+$ T cells were surprisingly recorded in the gut (MesLN). *In vitro* restimulation with influenza virus infected irradiated stimulators was also carried out on these cells to determine if it was possible to enhance any low frequency populations. Cells were stimulated with equal numbers of irradiated influenza virus-infected splenocytes for 7 days prior to tetramer staining. Culture of the cell suspensions with stimulators was unsuccessful and no tetramer positive cells were observed upon FACS analysis. IFNγ ELISPOT is considered to be a superior technique for identifying and enumerating low frequency populations, so all future $CD8^+$ T cell frequency analysis focused on the use of ELISPOT for the identification of influenza virus-specific cells in the various tissues examined.

The results in Figure 8 show the virus-specific IFNγ secreting $CD8^+$ T cell response to influenza virus infection over time. Influenza virus infected mice were followed for up to 6 months post infection at which time memory cells were observed. The early response was dominated by recruitment of large numbers of virus specific $CD8^+$ T cells to the lungs. The response in the lung peaked at day 10 after which the cells rapidly dissipated. The response initially peaked in the MedLN (Day 7), but virus specific IFNγ secreting $CD8^+$ T cells were also detected in the spleen and surprisingly in the MesLN. There was also a substantial recruitment of virus-specific cells to the D-NALT and less surprisingly to the spleen.

3.2.3 Isoelectric Focusing of Antibodies: A Useful Tool to Investigate the Clonality of a Serum Humoral Response to Influenza Virus Infection

Isoelectric Focusing (IEF) is an established technique for separation of proteins with small differences in charge (Braun et al., 1979). The possibility of using IEF as a technique to separate antibodies generated during an immune response and to identify the clonality of the antibodies was investigated.

Gel running apparatus from two different manufacturers were evaluated for suitability as systems to separate antibodies following influenza virus infection. The first apparatus was the BioRad Criterion™ System which was compared to the PhastSystem™ from Amersham Biosciences. Both were assessed for ease of use and results obtained following protein staining and immunoblotting.

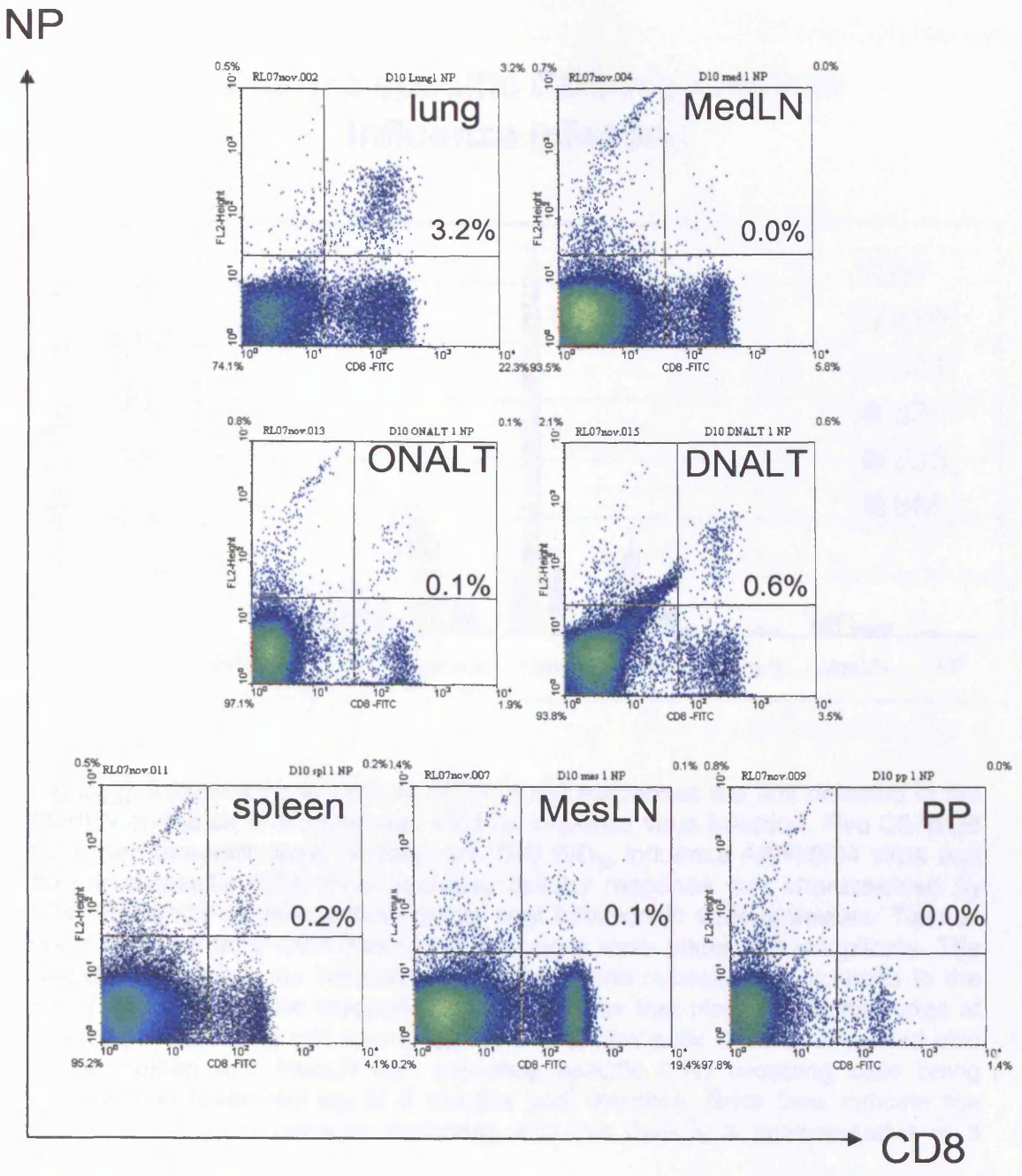


Figure 7: Direct influenza NP tetramer and CD8 staining on C57BL/6 tissues 10 days following intranasal influenza virus infection. Virus specific $CD8^+$ cells can been seen at various frequencies in all of the tissues examined except PP. The strongest response is seen in the lungs and D-NALT with 3.2% and 0.6% of the cells respectively being $CD8^+$ $NP_{366-374}$ H2-Db $tetramer^+$. At this timepoint, a minimal response is observed in the MedLN, which is below the level of detection of the assay.

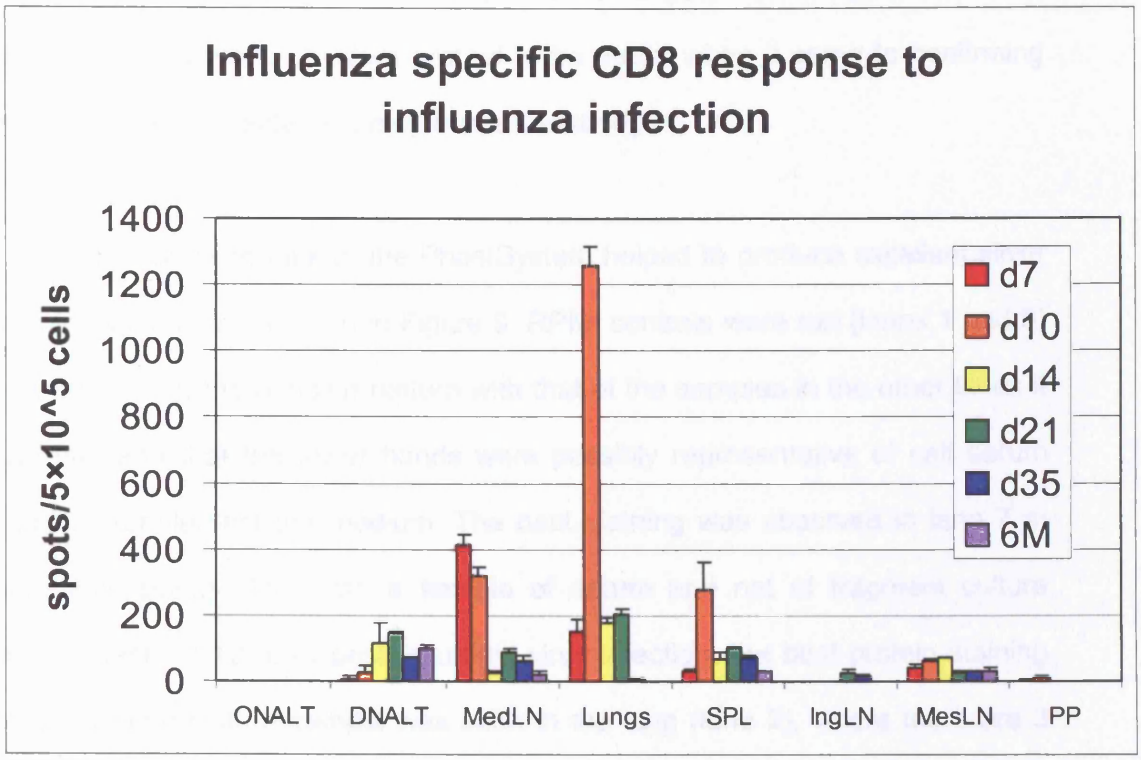


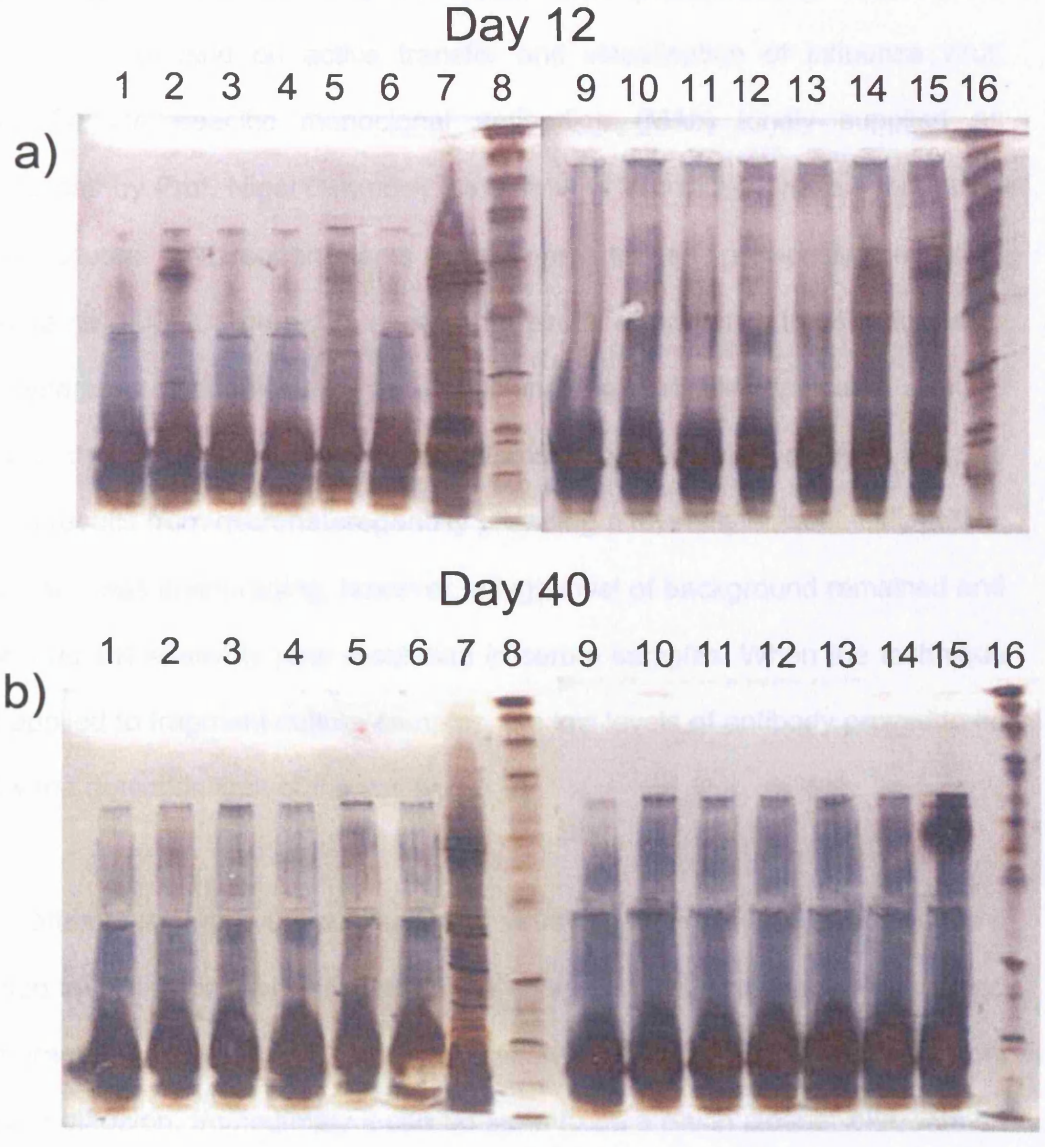
Figure 8: Influenza virus specific $CD8^+$ T cell responses are first detected in the MedLN and peak in the lung day 10 after influenza virus infection. Five C57BL/6 mice per timepoint were infected with 500 EID_{50} influenza A/PR/8/34 virus and the virus-specific $CD8^+$ IFNγ secreting cellular response was characterised by IFNγ ELISPOT at various time points post infection in various tissues. Tissues were pooled from 5 mice and cell suspensions were plated out in triplicate. The response seems to be initiated in the MedLN and subsequently spreads to the lungs and spleen. The response is dominated by that observed in the lungs at day 10 post infection and seems to persist predominantly in the D-NALT but also in the spleen and MesLN with influenza specific IFNγ secreting cells being observed in these tissues at 6 months post infection. Error bars indicate the standard deviation between replicates and this data is a representative of 3 experiments.

Both systems demonstrated strengths and weaknesses. In respect of ease of use and protein staining the PhastSystem was superior in terms of resolution. However, the Criterion system proved to be better when it came to confirming that the protein detected in samples was antibody.

The in-built staining tank of the PhastSystem helped to produce excellent silver staining which can be seen in Figure 9. RPMI controls were run (lanes 1 and 9) and comparing the banding pattern with that of the samples in the other lanes it can be seen that the lower bands were possibly representative of calf serum used to supplement the medium. The best staining was observed in lane 7 at both time points. This was a sample of serum and not of fragment culture supernatant. At 12 days post influenza virus infection, the best protein staining of a fragment culture sample was seen in the lung (lane 2), where there are 3 close bands which could represent 3 antibody forming cell clones. Interestingly, there is a weak but broad response seen in the D-NALT (lane 5) where there were 7 (uniformly spaced) bands observed on the freshly stained gel (but perhaps not as clear on the reproduced image). As the infection is cleared and the animals recover, the profile of protein staining of the fragment cultures and serum changes (Figure 9 b). The protein staining seen in the respiratory compartment vanishes and the clearest separation was observed in lane 15 where the spleen fragment culture was focused. There was also a noticeable banding pattern observed from the colon (lane10) but this has not reproduced as distinctly in the scan compared with the bands observed in the freshly stained gel.

Figure 9: Fragment cultures of C57BL/6 tissues were prepared following intranasal influenza A/PR/8/34 virus infection and separated by IEF on the PhastSystem and subsequently silver stained. Lane numbers correspond to fragment cultures taken from various tissues at either a) the peak of infection or b) 40 days post infection, the key can be seen below. Lane 2 in both images shows RPMI medium containing foetal bovine serum which was used in the fragment culture method and is thought to contribute to the large band observed at the bottom of the gels. At day 12 post infection (a) the best protein staining is seen in the serum. There is also a concentration of protein seen in the lungs (lane 2). By day 40 post infection, no protein staining is observed in the respiratory compartment. The best staining is seen in the spleen (lane 15), with some banding also observed in the lamina propria.

1	RPMI	9	RPMI
2	Lung	10	Colon LP
3	MedLN	11	ileum LP
4	MedLN	12	PP
5	DNALT	13	MesLN
6	ONALT	14	BM
7	Serum	15	Spleen
8	pI markers	16	pI markers


Day 12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
a)
Day 40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
b)

The silver staining method simply stains protein so attention was turned to trying to identify influenza virus-specific immunoglobulin bands in the samples. The blotting system supplied with the PhastSystem proved to be very inefficient at transfer, so the BioRad Criterion system was predominantly used. Initial experiments focused on active transfer and visualisation of influenza virus A/PR/8/34 HA specific monoclonal antibodies (MAb) kindly supplied as hybridomas by Prof. Nigel Dimmock (University of Warwick). These hybridomas were cultured and supernatants were prepared as previously described (Yokoyama, 2000). Figure 10a shows the results of an active transfer following IEF separation of the MAbs. Two monoclonals in particular (indicated using *) showed the characteristic banding patterns expected. This multiple banding pattern results from microheterogeneity providing a diversity of isoelectric points. This result was encouraging, however, a high level of background remained and there was still relatively poor resolution in serum samples. When the technique was applied to fragment culture samples, the low levels of antibody proved to be below the detection limit of the assay.

In an attempt to improve visualisation of virus-specific immunoglobulin bands a method involving passive diffusion of the antibodies onto influenza virus coated membranes was assessed. Figure 10b shows the initial results obtained from passive diffusion. Immediately it can be seen to be a much cleaner blot, with no background, and no bands present in the negative control (naïve serum). Few bands were observed at day 15 post influenza virus infection, but there appears to be a large amount of smearing within the lane. Perhaps this is indicative of a more polyclonal response shortly after the peak of infection. The results from 60 days post infection show that there are several more distinct bands, perhaps indicative of greater levels of antibody production which could be an efficient system for controlling subsequent infection.

The method of passive transfer was applied to fragment cultures but no bands could be visualised. The level of antibody in the fragment culture samples appeared to be below the level of detection of this method. Attempts were made to concentrate the fragment cultures approximately 10-fold using Vivaspin columns. However, this had no effect on increasing the sensitivity of immunoglobulin visualisation.

Unfortunately, time constraints prevented further development of IEF analysis at this stage. The technique that was developed could be applied to monitor the clonality of a serum antibody response to influenza virus infection, and perhaps be adapted to any antigen encounter. The main goal of the IEF study was to characterise the antibody response from within the various tissues to give a snap shot of the AFCs in circulation at any one time. It was hoped that this technique would provide some information about the AFCs in specific tissues and an insight into the breadth of clonal response (number of bands) and strength of response (size of bands) and the circulation of these cells following a sterilising immune response. These could be important factors when assessing the efficacy of a vaccine candidate.

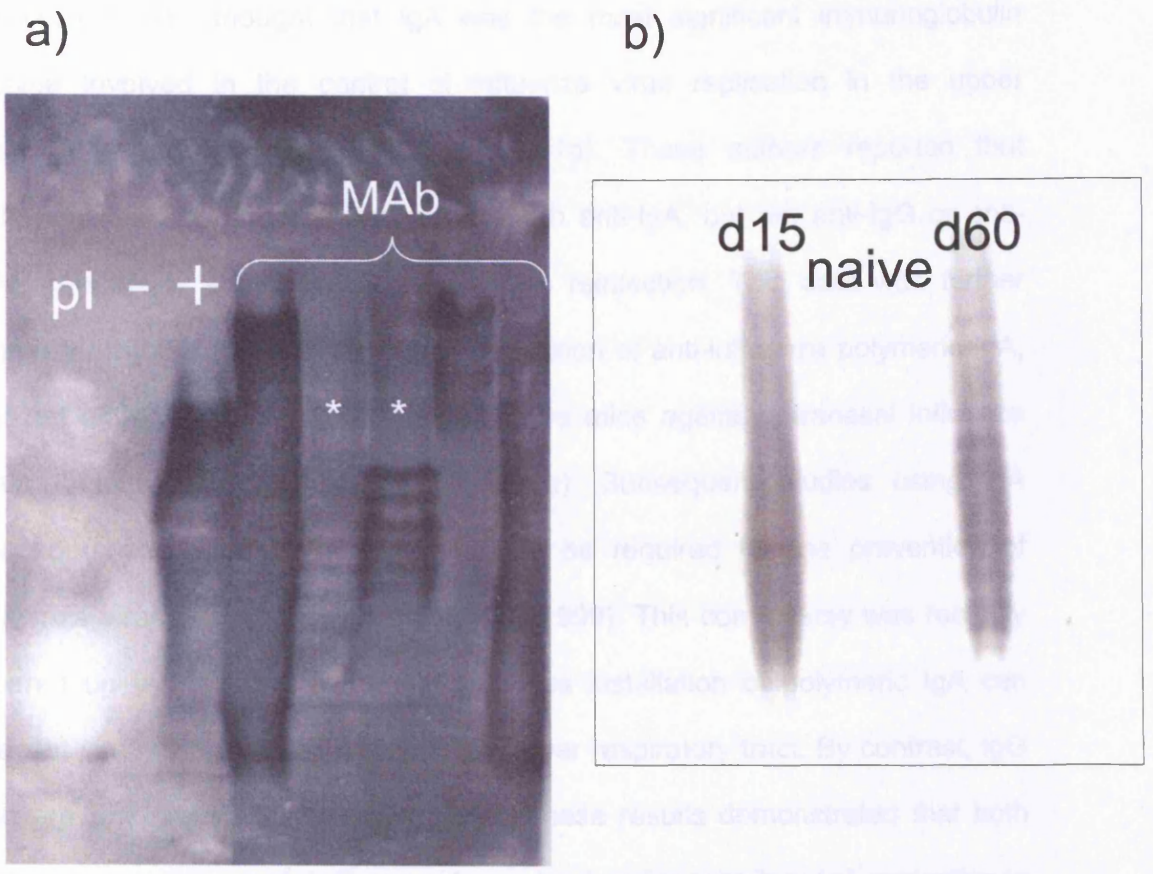


Figure 10: a) active influenza virus-specific immunoblotting following monoclonal antibody isoelectric focusing using the BioRad Criterion system. The image shows the lanes where isoelectric point (pI) makers, naïve serum (-), and serum from an influenza virus infected mouse (+) were focused. * indicate characteristic banding patterns of MAbs. b) native transfer of antibodies from naïve serum and serum collected on days 15 and 60 post influenza virus infection. At day 60 post infection, several bands are observed possibly characteristic of a polyclonal response more efficient at controlling subsequent infection.

3.3 Discussion

Originally it was thought that IgA was the most significant immunoglobulin isotype involved in the control of influenza virus replication in the upper respiratory tract (Renegar & Small, 1991a). These authors reported that following infection, intranasal treatment with anti-IgA, but not anti-IgG or anti-IgM, completely abolished protection from reinfection. This data was further supported by the fact that intravenous injection of anti-influenza polymeric IgA, but not anti-IgG or IgM, could protect naïve mice against intranasal influenza virus infection (Renegar & Small, 1991b). Subsequent studies using IgA knockout mice proved that IgA may not be required for the prevention of influenza virus infection (Mbawuike et al., 1999). This controversy was recently cleared up by finding that only intravenous installation of polymeric IgA can prevent virally induced pathology in the upper respiratory tract. By contrast, IgG can prevent viral pathology in the lung. These results demonstrated that both isotypes were important. IgG can act as a backup for secretory IgA protection in the nose, as well as being the isotype involved in protection in the lung (Renegar et al., 2004).

Unfortunately use of the fragment culture technique to isolate virus-specific antibodies did not allow quantification of anti-influenza specific IgA in the D-NALT. IgA AFCs appear to be very sensitive to the fragment culture conditions because this was the only isotype to remain undetected in the D-NALT. Previous studies by our group have shown that IgA AFCs are the most numerous and long lived cells remaining within or being constantly recruited to the D-NALT (Liang et al., 2001). Further studies within our group have shown a requirement for lower pressure sorting of AFCs by FACS to ensure continual antibody secretion and lack of survival of these cells in adoptive transfers which also hints

at the sensitivity of these cells to manipulation (Dr S Hou, personal communication).

In cellular responses to respiratory virus infections dendritic cells (DCs) have been shown to play a role in initiating and driving T cell responses to infections in the lungs (Banchereau & Steinman, 1998). Resident lung DCs acquire antigen from the invading virus, become activated and subsequently traffic to the local draining lymph node (mediastinal). It is here where the antigen can be displayed to naïve T lymphocytes resulting in their activation, clonal expansion and subsequent migration. This has been clearly demonstrated for $CD8^+$ T lymphocytes becoming activated in influenza virus infection (Cerwenka et al., 1999a) and it is understood that $CD8^+$ cytotoxic T cells play a key role in viral clearance (Doherty et al., 1997, Swain et al., 2004). Neutralising antibody plays a major role in protection from re-infection and immune memory, but does not contribute significantly in viral clearance during a primary infection unless the viral load is high (Eichelberger et al., 1991a, Epstein et al., 1998). The ELISPOT data (Figure 8) shows a strong early response in the MedLN which appears to have peaked by day 7. This coincides with a sudden influx of $CD8^+$ effector T cells into the lungs, peaking at day 14 before rapidly tailing off. Such an accumulation of $CD8^+$ T cells in the lungs correlates with an expected clearance of the virus by day 10 post infection (Doherty et al., 1997). The T-lymphocyte mediated viral clearance has been shown to involve Fas or perforin dependent mechanisms (Topham et al., 1997).

The initial response seen in the MedLN was also observed in a study of activation, differentiation and migration of influenza virus specific $CD8^+$ T cells during influenza virus infection (Lawrence & Braciale, 2004). The authors report an initial activation of $CD8^+$ T cells in the MedLN that is exclusive to the draining

lymph node and occurs during the first three days post infection. The activated MedLN cells were shown to exit the draining lymph node and traffic to and seed the spleen and peripheral lymph nodes. The most highly activated and divided cells were found to traffic to the lungs. These observations correspond with the observations from the data reported here and provide an explanation for the initial response seen in the MedLN which spreads rapidly to the lungs, spleen and MesLN.

This data shows significant virus-specific effector $CD8^+$ T cell populations in the spleen, D-NALT and more surprisingly the MesLN. In murine influenza virus and Sendai virus infection models, the response observed in the spleen has been explained by the distribution of antigen presenting cells (Flynn et al., 1998, Usherwood et al., 1999b). A recent study has also shown the preferential migration of effector $CD8^+$ T cells, induced both by viral and bacterial infection, to non-lymphoid tissues (Masopust et al., 2001) perhaps explaining the effector $CD8^+$ T cells observed in the D-NALT. There is also evidence that T cell responses to influenza do not require encapsulated lymph nodes and that the NALT is sufficient for inducing immune responses (Lund et al., 2002). The reported study used lymphotoxin-alpha deficient mice which lack lymph nodes and have a disrupted splenic architecture. These mice were observed to have a capacity to elicit delayed but strong influenza virus-specific $CD8^+$ T cell responses that were capable of killing target cells displaying influenza viral peptides. The mice were able to control low titre infections of influenza virus. However, mice succumbed to a high dose infection resulting from the delay in the generation of effector $CD8^+$ responses. This demonstrates that essential organised lymphoid tissues such as lymph nodes or the spleen are not absolutely required to generate effective immunity against influenza virus infection.

In contrast to previous reports regarding the distribution of antigen presenting cells and the resultant response seen in the spleen, data generated by Lawrence and Braciale (2004) showed activated MedLN cells exiting the draining lymph node before trafficking to and seeding the spleen and peripheral lymph nodes. These observations correspond with the observations from the data reported here and provide an explanation for the initial response seen in the MedLN which spreads rapidly to the lungs, spleen and MesLN. Considering both data, perhaps the initial response observed in the spleen is as a result of both MedLN cells and APCs trafficking there.

Following recovery from acute infection a state of immunological memory develops which functions to protect from reinfection. The data produced indicates that at 6 months post infection influenza-specific $CD8^+$ T cells persist in the D-NALT, MedLN, MesLN and the spleen. The populations observed in the lymph nodes and some of the population in the spleen are likely to be central memory (T_{CM}) populations due to their location, and the requirement of CD62L expression for lymph node homing (Gallatin et al., 1983). They may however also be T_{EM} populations circulating from the tissues. By contrast, it is possible that the populations observed in the D-NALT, and to a lesser extent the spleen, are effector memory (T_{EM}) populations (Sallusto et al., 1999) which have the ability to migrate to non-lymphoid tissues, a characteristic of T_{EM} cells generated through viral or bacterial infection (Masopust et al., 2001). T_{EM} cells are an essential and powerful first line defence, whereas the T_{CM} populations provide reserve defences (Lanzavecchia & Sallusto, 2002). There is evidence that T_{CM} and T_{EM} are generated differentially during and immune response, depending on the conditions of activation (Manjunath et al., 2001, Weninger et al., 2001). These authors report that murine $CD8^+$ T cells activated *in vitro* can acquire the characteristics of either T_{CM} or T_{EM}. By contrast, it has also been proposed that

T_{CM} and T_{EM} do not necessarily represent distinct populations but are in fact part of a differentiation pathway in which naïve T cells are activated into effector cells, before differentiation into T_{EM} followed by the acquisition of T_{CM} characteristics (Wherry et al., 2003). Wherry *et al.* (2003) investigated the appearance of $CD8^+$ T_{EM} and T_{CM} after infection of mice with lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes*. The authors report that $CD8^+$ T_{EM} convert to T_{CM} and proposed the linear differentiation model for the generation of $CD8^+$ T_{EM} and T_{CM}. The model describes T_{EM} being derived directly from effector cells and T_{CM} derived from T_{EM}. Whether the population of memory cells observed in the D-NALT are in fact T_{EM} cells remains to be investigated.

To compare and support the data generated in this study, Weninger *et al.* (2001) demonstrated that murine antigen primed $CD8^+$ T cells cultured *in vitro* with interleukin (IL)-15 resemble T_{CM} cells in phenotype and function, while cells stimulated with IL-2 resemble T_{EM} cells. In transfer studies, it was shown that naïve and T_{CM}, and to a lesser extent T_{EM} cells localised to the T cells areas in the spleen, whereas only T_{CM} cells homed to the lymph nodes.

The $CD8^+$ T cell migratory pattern was recently characterised following secondary challenge with influenza A virus in mice that had already been primed with a serologically distinct influenza A virus (Marshall et al., 2001). The authors report populations of memory $CD8^+$ T cells that are phenotypically diverse and widely dispersed with the largest numbers of influenza virus-specific $CD8^+$ T cells detected in the spleen at every timepoint and the response peaking at day 10 post challenge.

Current influenza vaccines in clinical use contain HA as their main or only viral antigen. The efficacy of the current vaccines depends primarily on the antigenic match between circulating viruses with the strains used for vaccination, as well as the subject's age and immune status. Characterisation of the humoral and cellular response to influenza virus infection has produced data that can be compared to the responses induced by vaccination. It is expected that for a vaccine to be efficacious it must mimic the natural protective response generated by influenza virus infection and that it should produce a strong virus-specific response in the lungs which will contribute to the killing of virus infected cells and thus limit or prevent the spread of viral infection.

The work described in the next chapter will focus on the response generated by a DNA vaccine administered by PMID of influenza virus NP DNA coated on gold particles. The plasmid used encodes a conserved antigen (NP) that does not vary between strains and is not subject to antigenic shift and drift. The study has also been developed to see if a non-mucosal route of immunisation can initiate similar $CD8^+$ T cell responses as experimental infection does, and more interestingly whether it can induce a response in the NALT, where antigen is first encountered, similar to the results observed in the experimental infection model.

Much of the literature published on IEF is concerned with IgG detection in samples collected from multiple sclerosis (MS) patients. Analysis of oligoclonal bands of IgG and IgM focused from cerebrospinal fluid using IEF gels is an established test used in the diagnosis of MS (Andersson et al., 1994, Sharief et al., 1990). However, there are some older papers that discuss the focusing and identification of antibodies (Braun et al., 1979, Keck et al., 1973). The paper by Keck *et al.* describes a technique using radiolabelled specific antigen to visualise antibodies within whole serum.

The most successful IEF technique from this study concentrated on the BioRad Criterion system. Influenza A/PR/8/34 virus-specific MAbs produced the characteristic banding patterns expected (figure 10a), which confirmed that a sensitive technique capable of visualising anti-influenza antibodies had been developed. However, concerns remained regarding the detection of immunoglobulin in the fragment cultures. When the technique was applied to fragment cultures it proved to be insufficiently sensitive to detect immunoglobulin. A second native transfer method improved resolution (figure 10b), however it also proved to lack the sensitivity required to detect the low levels of antibody in the fragment cultures.

Various problems were encountered whilst trying to set up the IEF technique. Difficulties ranged from poor reagents and technical support to identifying bands on gels to be immunoglobulin. Various protein stains were investigated, and silver staining gave the best result using the Amersham PhastSystem (Figure 9). When this system was used for western blotting techniques however, it was found to have a very poor blotting system. The most successful IEF technique from this study concentrated on the BioRad Criterion system. Influenza A/PR/8/34 virus-specific MAbs produced the characteristic banding patterns expected (figure 10a), which confirmed that a sensitive technique capable of visualising anti-influenza antibodies had been developed. However, concerns remained regarding the detection of immunoglobulin in the fragment cultures. When the technique was applied to fragment cultures it proved to be insufficiently sensitive to detect immunoglobulin. A second native transfer method improved resolution (figure 10b), however it also proved to lack the sensitivity required to detect the low levels of antibody in the fragment cultures.

Low sensitivity led to the investigation into concentration of fragment cultures using Vivaspin columns to concentrate fragment cultures approximately 10-fold. Unfortunately, concentration using this method had no effect on increasing the sensitivity of the technique. If time had allowed, it may have been interesting to try and isolate influenza virus-specific antibodies. It could be possible to do this by using affinity column purification. Amersham Biosciences manufacture HiTrap Affinity columns to which it would be possible to couple purified influenza virus. Fragment cultures could then be run through the column, and influenza virus-specific antibodies would be retained in the column bound to the purified virus. The virus bound antibodies could then be eluted with an alkaline solution before being focused on IEF gels.

Another possible alternative would be to create a virus-specific hybridoma library in a similar style to the work carried out by J.E. Crowe Jr. (Crowe et al., 1994). This study involved the immunisation of chimpanzees with recombinant vaccinia virus expressing respiratory syncytial virus (RSV) F or G protein. Animals were then challenged with RSV and peripheral blood lymphocytes isolated. Total lymphocytes were then transformed with Epstein-Barr virus to generate lymphoblastoid cells lines that secreted anti-RSV antibodies. All lymphoblastoid cells generated were screened for anti-RSV production by ELISA, and supernatants were tested for viral neutralisation. To adapt this technique for this study, cell suspensions would have to be prepared from all of the tissues investigated and hybridoma cell lines generated. Once anti-influenza virus antibodies were identified, the CDR3 regions of the binding site of an antibody could then be determined by sequencing and compared to the CDR3 regions of antibodies produced in other compartments. This would identify whether the same antibody is produced in different areas of the body and therefore whether

antibody secreting cells from the same clone have trafficked to different parts of the immune system to produce the same anti-influenza virus antibody.

IEF has been shown to be a novel method for the characterisation of the clonality of a serum antibody response to influenza virus infection. This method has the potential to be applied to monitor the development of the response in individual mice as influenza virus infection is established and then cleared before the establishment of memory. The fragment culture technique developed provided an excellent way to collect antibody from various tissues of the mucosal and systemic immune systems. It proved sufficient for ELISA based analysis but not IEF. More recently, B cell clonality has been investigated by PCR methods and has concentrated on the detection of lymphoma. Perhaps an alternative route of investigation to take would be the development of PCR assays designed to detect the unique variations in the Ig heavy regions of specific antibodies as previously described (Kusic et al., 2003, Leal et al., 2003, Zompi et al., 2004).

Chapter 4

Characterisation of the Immune Response to Particle Mediated Immunotherapeutic Epidermal Delivery of a DNA Vaccine

4.1 Introduction

Influenza DNA vaccines have been extensively studied and constructs encoding the surface glycoproteins, HA and NA, internal proteins NP and M1 and non structural protein NS1 have all been assessed for their ability to generate protective immune responses (Chen et al., 1998, Robinson et al., 1997, Ulmer et al., 1993a). Immunisation with plasmid DNA encoding influenza HA is capable of inducing cell-mediated and humoral immunity and protecting mice against intranasal challenge with influenza virus (Ulmer et al., 1994, Webster et al., 1994). Furthermore, induction of CTLs and protection against virus challenge has been demonstrated following immunisation with plasmid DNA encoding influenza NP (Ulmer et al., 1993b). These studies have primarily been carried out by introducing the DNA by intramuscular injection, and research into gene gun immunisation or particle mediated immunotherapeutic delivery (PMID) of DNA has been limited and less productive (Chen et al., 2000).

The aims of this study were to assess particle mediated immunotherapeutic delivery (PMID) as a method of DNA immunisation by comparing the immune response generated to that of the "gold standard" (infection that results in protection from reinfection) already characterised for influenza virus infection of mice in chapter 4. More specifically, there was interest to determine whether non mucosal PMID of influenza virus NP DNA was able to generate a mucosal response in the D-NALT.

4.2 Results

4.2.1 Cartridge Preparation, Quality Control and Immunisation Regimen

Preparation of cartridges for PMID using the BioRad Helios research device was carried out as previously described (section 2.14) and cartridges were tested for a uniform firing pattern by layering one sheet of mylar over a layer of parafilm. Mylar was suggested by PowderJect/Chiron to act as a substitute "skin-like" membrane to monitor penetration of plasmid coated gold beads through the Mylar and into the parafilm. Figure 11a shows a typical mylar/parafilm membrane following test firing and a poor firing pattern is seen in Figure 11b. Densitometry was carried out using a BioRad Densitometer to ensure even penetration of the gold beads. A representative scan showing a consistent firing pattern can be seen in Figure 11a. This is reflected in the similar size and intensity of gold colour of spots observed on the parafilm. Most firing patterns were like that observed in Figure 11a. If a poor firing pattern was observed the gene gun was primed with helium by firing a few "empty shots" and the cartridge batch was retested. If the firing pattern remained poor the batch was failed and the cartridges were remade.

The plasmid used contained the whole influenza virus NP gene inserted into the pVac vector. The details of the pVac-NP vector are shown in Figure 12a. The plasmid contains a cytomegalovirus intermediate early promoter to enable transcription of the NP gene, an origin of replication for growth in *E.Coli*, and ampicillin resistance which was used for selection. Results of a simple digest of the plasmid to show the presence of the whole NP gene in pVac-NP at around 1600 bps is shown in Figure 12b.

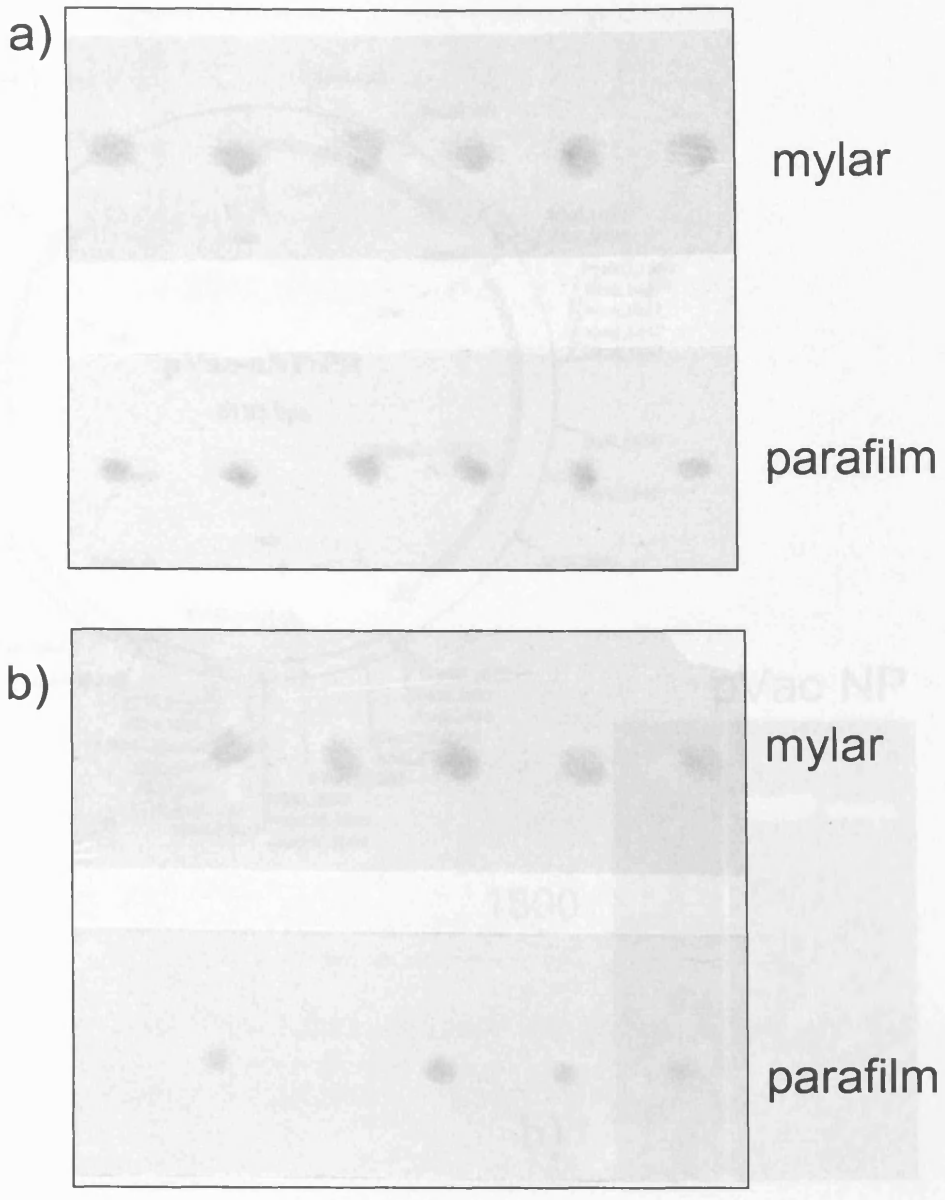


Figure 11: Freshly prepared DNA cartridges were test fired through mylar layered over parafilm a) shows a typical even firing pattern. Gold particles that travel through the mylar do so at an even rate for each shot and this is reflected in the even size and intensity of colour of the spots on the parafilm b) shows an uneven firing pattern where one shot has almost failed to penetrate the mylar and others vary in their colour density and size.

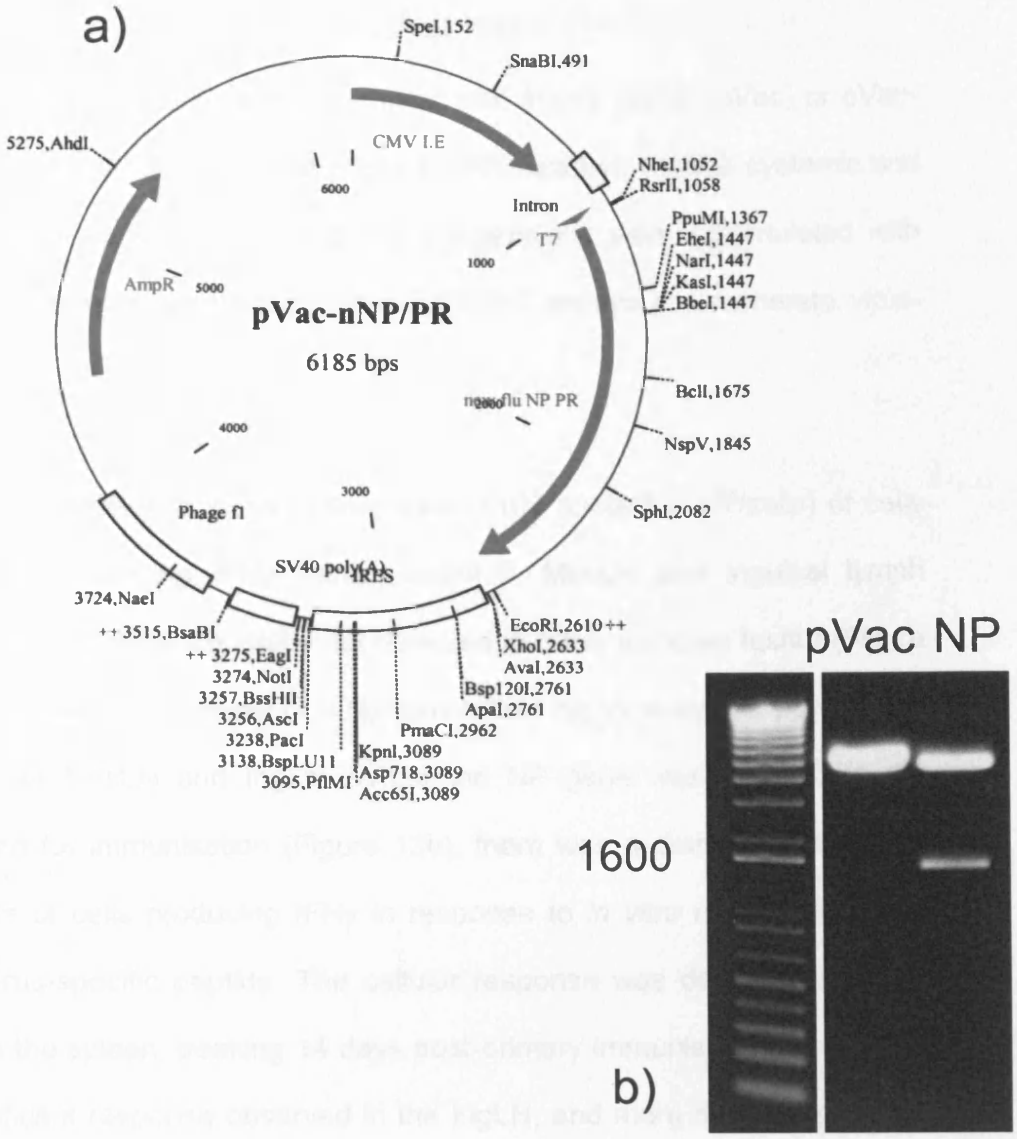


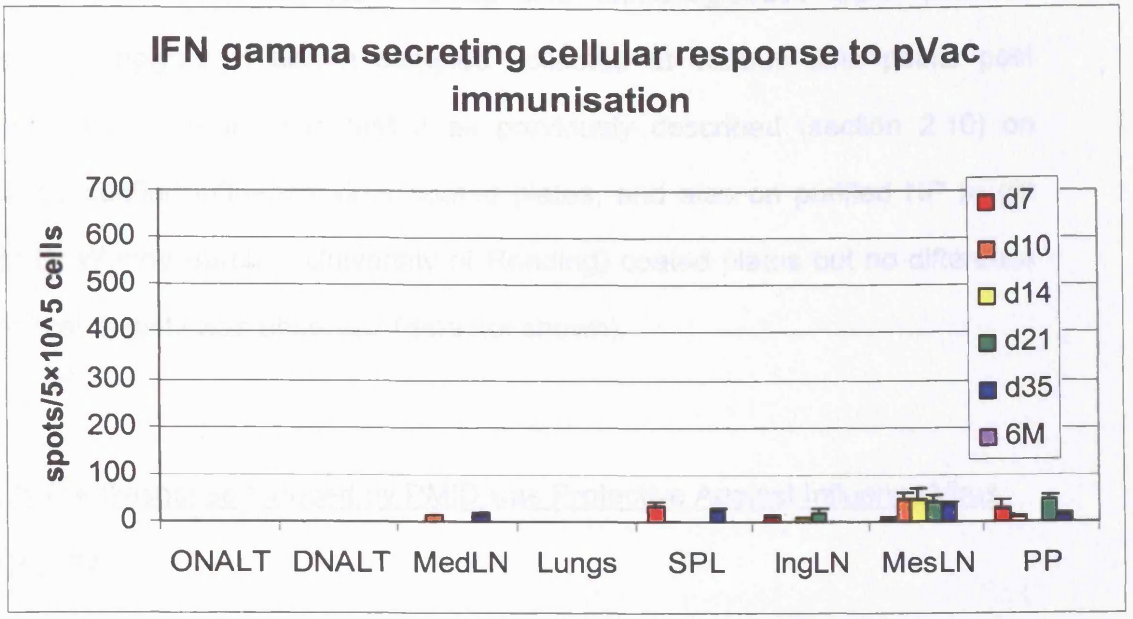
Figure 12: a) plasmid map of pVac containing the whole influenza virus A/PR/8/34 nucleoprotein (NP) gene, a promoter and ampicillin resistance for selection b) shows a simple digest of the plasmid to confirm the whole NP gene, roughly 1600 bps, is present .

4.2.2 PMID of Influenza Virus Nucleoprotein Induces a Negligible Humoral Response but a Long Lived $CD8^+$ T Cell Response

In initial experiments, mice were immunised with empty vector (pVac) or pVac-NP and at several time points post primary immunisation, various systemic and mucosal tissues were removed and cell suspensions were restimulated with influenza virus-specific peptide in IFNγ ELISPOT assays to enumerate virus-specific $CD8^+$ T cells.

In the pVac immunised animals, a low level (<100 spots/5 x 10^5/cells) of cells spontaneously producing IFNγ were present in MesLN and inguinal lymph nodes (IngLN), but were generally not detected in other samples tested (Figure 13a). The spontaneous release of IFNγ may reflect highly activated populations of cells in the MesLN and IngLN. When the NP gene was included in the plasmid used for immunisation (Figure 13b), there was a dramatic increase in the numbers of cells producing IFNγ in response to *in vitro* restimulation with influenza virus-specific peptide. The cellular response was dominated by that observed in the spleen, peaking 14 days post-primary immunisation. There was also a significant response observed in the IngLN, and more surprisingly in the D-NALT and MedLN. There were a low number of virus-specific cells recruited to the lungs with a slower kinetics. At 6 months post immunisation the results indicated that there were populations of virus-specific IFNγ secreting memory lymphocytes observed predominantly in the spleen with lower numbers circulating to the periphery that were still detectable in the MedLN, IngLN and MesLN.

a)



b)

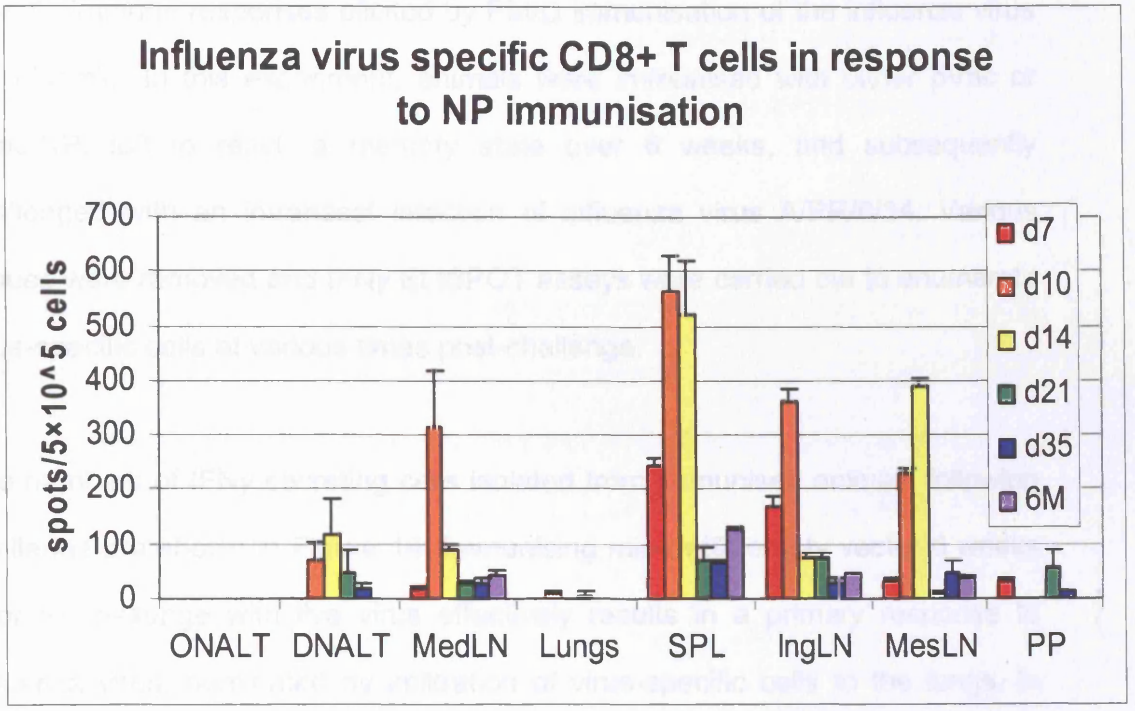


Figure 13: Influenza virus-specific CD8⁺ T cells responses are first detected in the spleen and IngLN and peak in the spleen day 10 after PMID immunisation. Five C57BL/6 mice per timepoint were immunised with 2 non overlapping doses of approximately 0.5μg pVac (a) or pVac-NP (b) DNA. Tisses were pooled from 5 mice per timpoint and cell suspension were plated out in triplicate. Virus-specific IFNγ secreting CD8⁺ T cells were were enumerated by ELISPOT. Background activated IFNγ secreting cells were observed in the IngLN and MesLN following pVac immunisation. pVac-NP immunisation generated a dominant response in the spleen, IngLN and MesLN. A significant response was also observed in the respiratory compartment. Error bars indicate the standard deviation between replicates and this data is representative of two experiments.

Minimal levels of influenza virus-specific immunoglobulin were detected following analysis of serum samples collected at various time points post immunisation. Serum was tested as previously described (section 2.10) on sucrose purified influenza virus coated plates, and also on purified NP (a gift from Dr Wendy Barclay, University of Reading) coated plates but no difference in optical density was observed (data not shown).

4.2.3 The Response Induced by PMID was Protective Against Influenza Virus Challenge

A study was set up to investigate if any protection was provided by the virus-specific immune responses elicited by PMID immunisation of the influenza virus NP plasmid. In this experiment, animals were immunised with either pVac or pVac-NP, left to reach a memory state over 6 weeks, and subsequently challenged with an intranasal infection of influenza virus A/PR/8/34. Various tissues were removed and IFNγ ELISPOT assays were carried out to enumerate virus-specific cells at various times post-challenge.

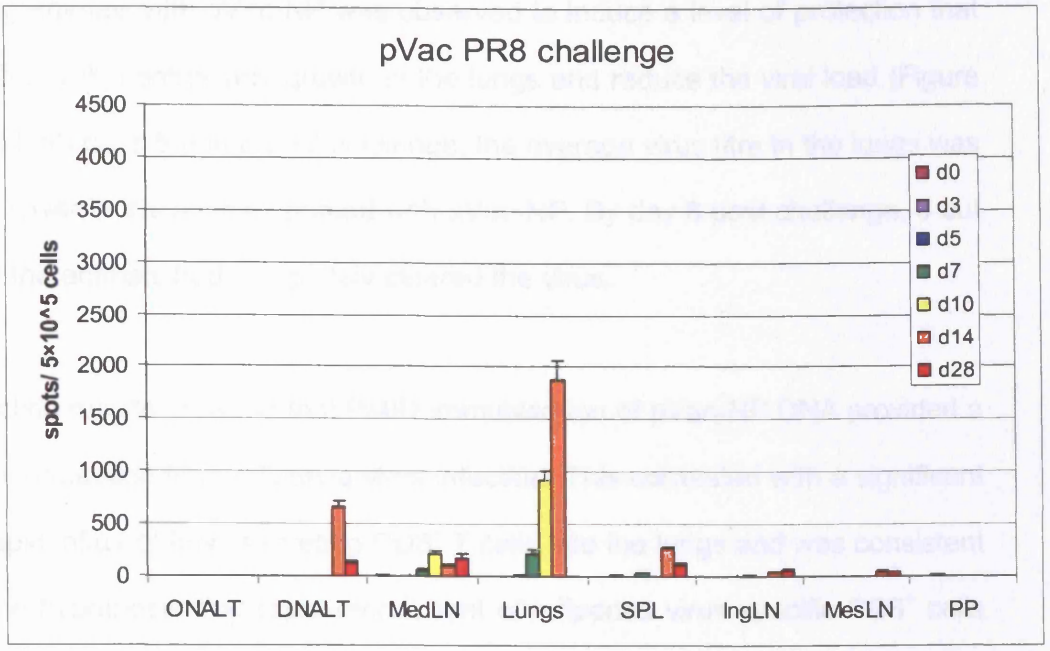
The numbers of IFNγ secreting cells isolated from immunised animals following challenge are shown in Figure 14. Immunising mice with empty vector 6 weeks prior to challenge with live virus effectively results in a primary response to influenza virus, dominated by infiltration of virus-specific cells to the lungs. In general, these results are consistent, within the bounds of experimental error, with those observed when the primary immune response was characterised by IFNγ ELISPOT. When animals are immunised with the pVac-NP plasmid the response is dramatically different. There is an overwhelmingly stronger and kinetically faster recruitment of virus specific IFNγ secreting $CD8^+$ T cells to the lungs first observed at 5 days post challenge, which is much earlier than seen in

a primary response. The level of cellular influx at day 7 is at the same level as seen at the peak of a primary response (day 10). This rapid recruitment of cells continues before peaking at day 14. The precise level of the peak response could not be quantitated as the spots were too numerous to accurately count. The recruitment of $CD8^+$ cells was first recorded in the MedLN followed by the lungs and spleen before finally a few virus-specific T cells are seen in the D-NALT.

Following the discovery that there was a rapid recruitment of virus specific IFNγ secreting $CD8^+$ cells to the lungs, it was of interest to investigate whether PMID immunisation of pVac-NP could induce any protection from influenza virus infection. The previous challenge experiment was therefore repeated but only lungs were collected at various days post challenge to determine virus titres.

Specific pathogen free 10 day old embryonated hen eggs were provided by The Institute for Animal Health (Compton, Berks) as a medium in which to propagate influenza infected lung homogenates as previously described (section 2.16). Briefly, lung homogenates were prepared and 10-fold serial dilutions were used to infect the allantoic cavity of the eggs in triplicate. Eggs were incubated at 37°C for 48 hours. The presence of influenza virus was detected by utilising the ability of the virus to agglutinate chicken red blood cells. The amount of infectious virus in a lung homogenate was estimated by measuring the highest dilution of homogenate that could still infect an embryonated egg. The smallest amount of virus capable of doing this, on 50% of occasions, is known as the egg infectious dose (EID_{50}). Virus titre in EID_{50} was calculated as previously described (Mahy, 1985).

a)



b)

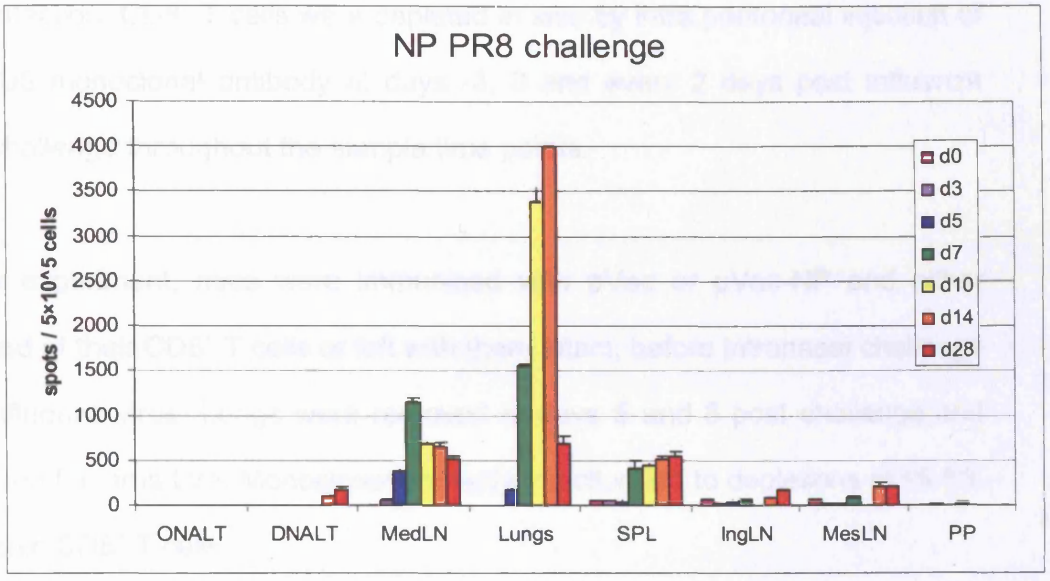


Figure 14: Infection with live influenza virus after PMID of pVac-NP results in the rapid infiltration of virus-specific $CD8^+$ T cells into the lung. Five C57BL/6 mice per timepoint were immunised with 2 non overlapping doses of approximately 0.5μg pVac (a) or pVac-NP (b) DNA before subsequent intranasal challenge 6 weeks later with 500 EID_{50} influenza A/PR/8/34 virus. Tissues were pooled from 5 mice per timpoint and cell suspensions were plated out in triplicate Virus-specific IFNγ secreting $CD8^+$ T cells were were enumerated by ELISPOT. pVac immunisation followed by challenge (a) resulted in a primary response to influenza. pVac-NP priming followed by challenge (b) was dominated by a strong and kinetically fast recruitment of virus specific IFNγ secreting cells to the lungs. Error bars indicate the standard deviation between replicates and this data is representative of two experiments.

Priming animals with pVac-NP was observed to induce a level of protection that can effectively control viral growth in the lungs and reduce the viral load (Figure 15). At both days 5 and 8 post challenge, the average virus titre in the lungs was 2 logs lower in the animals primed with pVac-NP. By day 8 post challenge, 3 out of 5 of the animals had completely cleared the virus.

Interesting results showing that PMID immunisation of pVac-NP DNA provided a level of protection from influenza virus infection. This correlated with a significant and rapid influx of IFNγ secreting $CD8^+$ T cells into the lungs and was consistent with the hypothesis that rapid recruitment of influenza virus-specific $CD8^+$ cells to the lungs could contribute to control of viral replication. In an attempt to determine the role of $CD8^+$ T cells in vaccine induced protection from influenza virus infection, $CD8^+$ T cells were depleted *in vivo* by intra peritoneal injection of anti-CD8 monoclonal antibody at days -3, 0 and every 2 days post influenza virus challenge throughout the sample time points.

In this experiment, mice were immunised with pVac or pVac-NP and either depleted of their $CD8^+$ T cells or left with them intact, before intranasal challenge with influenza virus. Lungs were removed at days 5 and 8 post challenge and assessed for virus titre. Monoclonal antibody injection led to depletions of 99.5% of splenic $CD8^+$ T cells.

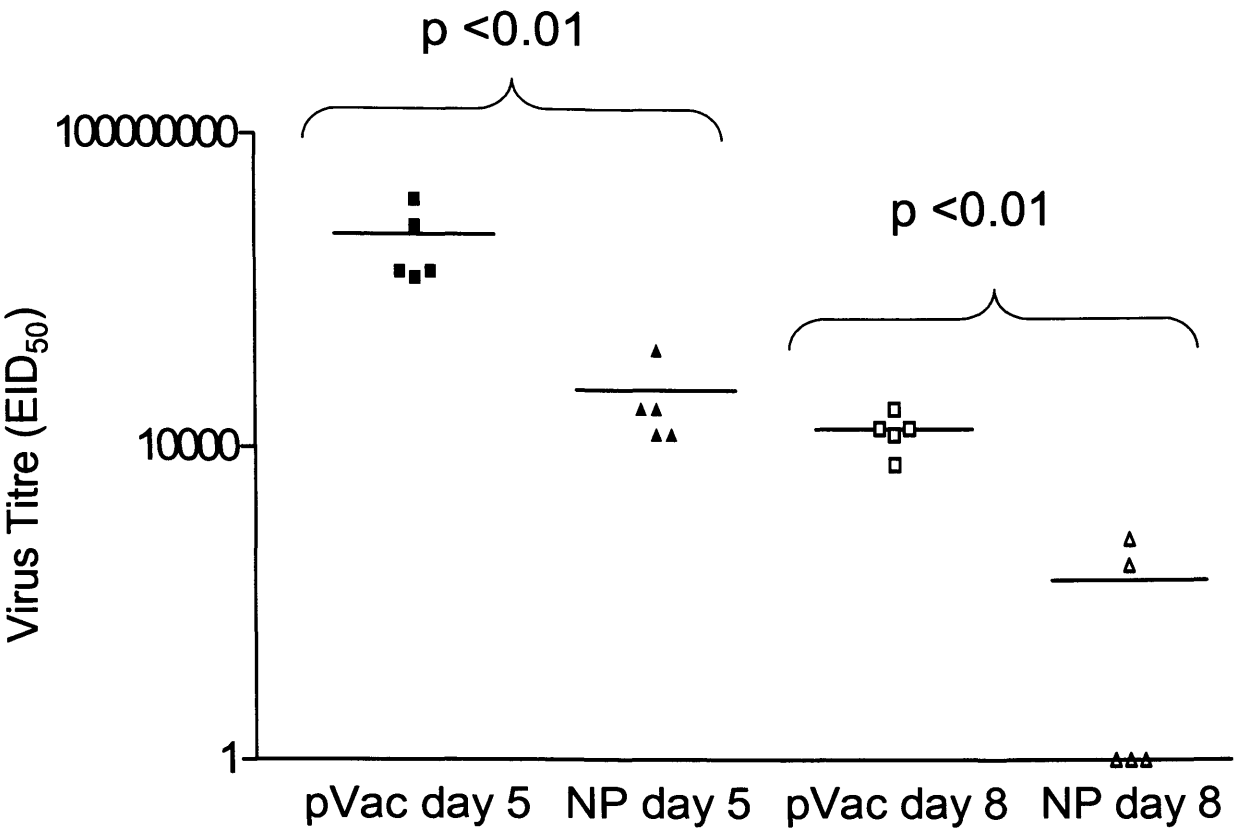


Figure 15: PMID of pVac-NP reduces virus titre in the lungs of influenza virus challenged mice. Five C57BL/6 mice per group were immunised with 2 non overlapping doses of approximately 0.5μg pVac or pVac-NP DNA before subsequent intranasal challenge 6 weeks later with 500 EID_{50} influenza A/PR/8/34 virus. Lungs were removed and virus titrations were carried out. At both days 5 and 8 post challenge, mice primed with pVac-NP showed a 2 log reduction in virus titre. By day eight, 3 out of 5 mice primed with pVac-NP had cleared the virus completely. Lung virus titres were analysed using the Mann Whitney test and NP immunisation is shown to significantly reduce viral growth in the lungs upon challenge. This data is representative of one experiment, with lung homogenates used to infect eggs in triplicate.

The lung viral titres recorded from the $CD8^+$ T-cell depletion experiment are shown in Figure 16. It can be seen that at day 5 post challenge (Figure16a) there is no effect of depleting the $CD8^+$ T cells. This result would be expected as the ELISPOT data indicated that the $CD8^+$ T cells are few in number and are only just beginning to arrive in the lungs by day 5 (Figure 14b). By 8 days post-challenge (Figure 16b) it can be seen that 2 out of 3 animals immunised with pVac-NP before the challenge, have already cleared the virus from their lungs and this has perhaps given a false suggestion that depleting the $CD8^+$ T cells has no effect on virus titre. The data generated from animals who were vaccinated with empty vector shows that depleting $CD8^+$ T cells has the effect of increading virus titre by one log. However, it is difficult to make any firm conclusions from this study, as, owing to time constraints this experiment was unable to be repeated. In retrospect, it would have been informative to determine the virus titres at day 7 after the challenge, before the pVac-NP immunised animals have had a chance to clear the virus.

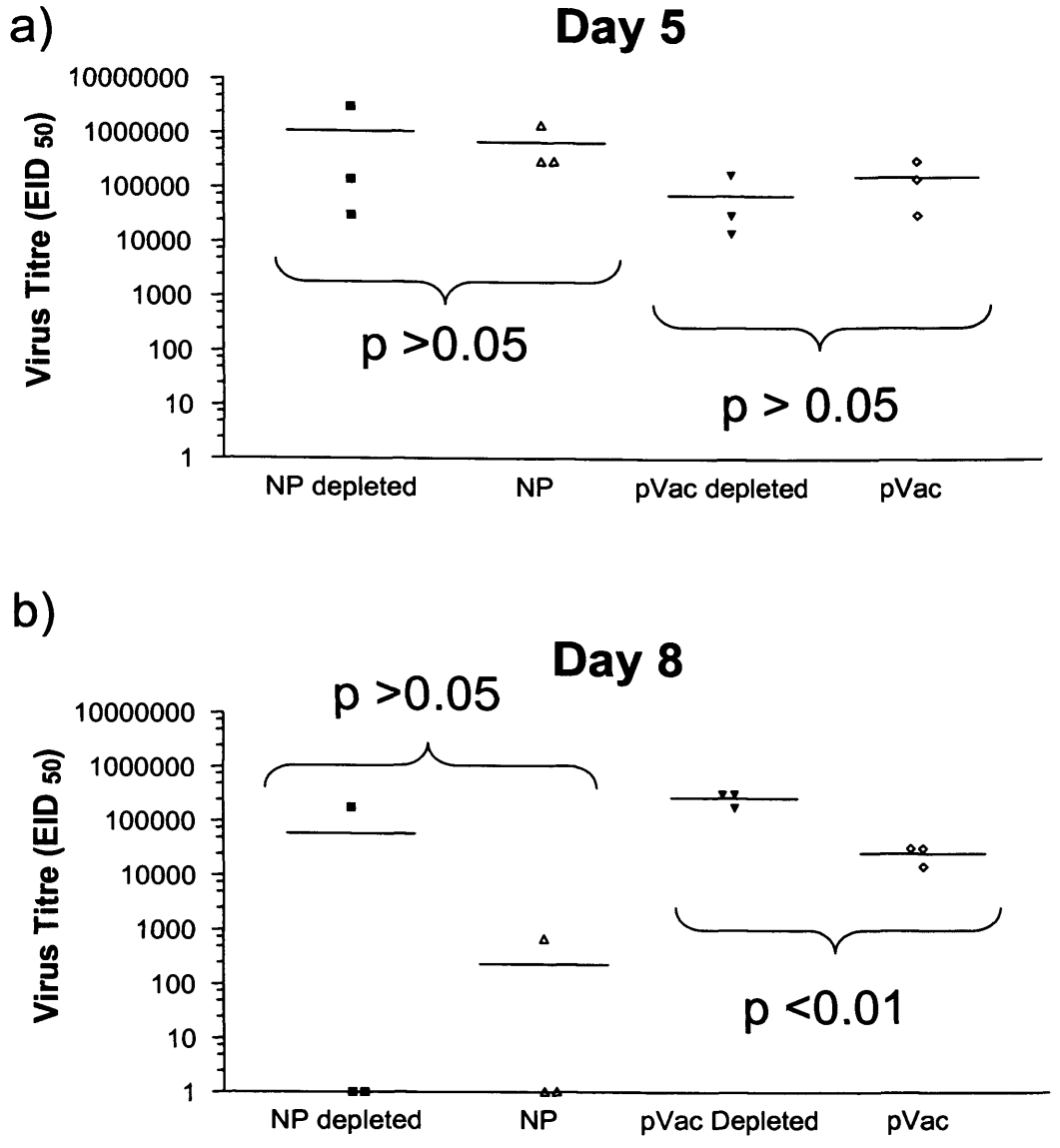


Figure 16: CD8 depletion of mice immunised with pVac-NP did not significantly affect virus clearance. Three C57BL/6 mice per group were immunised with 2 non overlapping doses of approximately 0.5μg pVac or pVac-NP DNA before subsequent intranasal challenge 6 weeks later with 500 EID_{50} influenza A/PR/8/34 virus. Mice were either depleted *in vivo* of their $CD8^+$ cells by monoclonal antibody, or left with them intact. Lungs were removed and virus titrations were carried out. At day 5 post challenge, $CD8^+$ cell depletion had little effect on lung virus titre. By day eight, 2 out of 3 mice in the groups primed with pVac-NP had cleared the virus and a conclusive interpretation of the results could not be made. The significance of viral load was analysed using analysis of variance followed by Tukey's test. This data is representative of one experiment, with lung homogenates used to infect eggs in triplicate.

4.3 Discussion

An important discovery in the field of DNA vaccination showed that the administration of plasmid vectors expressing human influenza virus protein resulted in protection in mice from disease following live influenza virus challenge (Fynan et al., 1993, Ulmer et al., 1993b). Intranasal, intramuscular and intravenous administration of plasmid DNA all resulted in protection to some extent but immunisation by gene gun generated by far the best results with as little as 0.4μg of DNA capable of inducing 95% protection. In mice it has been shown that approximately 100 fold less DNA is required to be administered by PMID to obtain a comparable response to that induced by needle injection (Fynan et al., 1993). DNA vaccines offer many advantages over conventional immunisation approaches: they are simple to make and deliver, they can elicit both humoral and cellular responses, and intracellular expression of native antigens imitates the route of viral pathogens. There is also the added bonus of being able to deliver multiple antigens/co-stimulatory molecules by combining several plasmids in one immunisation.

This study focused on assessing the response generated by PMID immunisation of influenza NP DNA with the view of comparing this to the natural protective response generated by intranasal influenza virus infection in mice. The aim was to elucidate information regarding the efficacy of PMID with regard to the generation of mucosal and/or protective immune responses.

ELISPOT was used to quantitate the number of NP-specific IFNγ producing $CD8^+$ T cells induced by a single PMID immunisation (comprising two non overlapping doses of approximately 0.5μg of DNA). The dominant cellular response is observed in the spleen and peaked at day 10 post immunisation.

There was also a substantial response in the draining lymph nodes of the skin, the IngLN. An early response was also observed in the MesLN, this was expected as a result of the immunisation method. Most interestingly, a virus-specific cellular response was observed in the respiratory compartment, namely the MedLN and more surprisingly the D-NALT. We believe that this is the first report of gene gun immunisation generating an antigen specific response in the D-NALT. Six months post immunisation, the memory response generated can be detected in the spleen, IngLN, MesLN and more surprisingly in the MedLN. Figure 13a shows the response observed when animals were immunised with an empty plasmid vector. There is a background response observed most significantly in the MesLN, this is however not surprising considering the insult on the gut by the immunisation method and its potential to cause inflammation and subsequently activate lymphocytes. It is however not observed six months later when a significant memory response is observed (Figure 13).

Memory cells located primarily at the sites of potential infection have the ability to respond quickly when exposed to a pathogen. Thus, according to their location, populations of memory T cells make a significant contribution to the first line of defense when a pathogen is encountered post vaccination. $CD8^+$ T cell memory can remain stable without a continuing requirement for additional exposure to antigen (Homann et al., 2001, Murali-Krishna et al., 1999). Therefore, a brief exposure to antigen should be sufficient to produce long-lived immunity. It has also been shown that the magnitude of the peak of effector $CD8^+$ T cell responses in the primary immune response can correlate to the extent of the long term memory induced (Hou et al., 1994). Together these data suggest that for a $CD8^+$ T cell inducing DNA vaccine to be the most effective, it should induce strong antigenic expression which results in a significant

expansion of $CD8^+$ T cells and leaves an expanded population of residual memory T cells.

As important as the generation of $CD8^+$ effector and memory cells, is the requirement for $CD4^+$ T cell help for the generation and maintenance of a good quality memory $CD8^+$ T cell response (Chan et al., 2001). These authors showed that MHC class II deficient mice were unable to mount a CTL response following DNA vaccination. It has been subsequently shown that $CD8^+$ T cells primed in the absence of $CD4^+$ T cell help may express a memory phenotype but are devoid of memory function (Bourgeois et al., 2002). These data have also been supported in the influenza model where $CD8^+$ T cell memory was found to be impaired in $CD4^+$ deficient mice (Belz et al., 2002). More recently several other studies have demonstrated a requirement for $CD4^+$ T cell help to establish fully functional $CD8^+$ T cell memory (Janssen et al., 2003, Sun & Bevan, 2003). $CD4^+$ T cells are required during the maintenance phase of memory development and not solely at the original $CD8^+$ T cells programming phase (Sun et al., 2004). These data suggest that when designing and selecting novel vaccine candidates it would be an advantage if they could induce both specific cytotoxic $CD8^+$ T cell and $CD4^+$ T helper cell responses.

Comparing the memory response generated by PMID of NP DNA (Figure 13) to that generated by natural infection (Figure 8), it would appear that natural infection has generated a superior memory response. Despite the ability of PMID to generate a primary response in the DNALT, immunised animals fail to maintain a memory response in this tissue. A stronger/longer-lasting memory response was observed in the D-NALT following natural infection.

Analysis of serum collected from PMID immunised animals showed that for this model antigen and method of immunisation there is a limited ability to generate humoral responses following a single immunisation. This result was surprising as there are several reports of single DNA immunisations generating robust cellular and humoral immune responses (Justewicz et al., 1995, Kwissa et al., 2000, Lodmell et al., 2002, Lodmell et al., 2003, Tollefsen et al., 2002). The coating antigen for the ELISA analysis of the serum was sucrose purified influenza virus A/PR/8/34 and it was possible that there was a problem with the antibodies in the serum recognising the conformation of the NP present in this preparation. Dr Wendy Barclay kindly provided some purified influenza virus A/PR/8/34 NP which was also tested as a coating antigen but again no specific antibodies were detected. These results indicate that it was likely that a negligible humoral response was generated to the single immunisation regimen adopted for these studies. A report investigating the immune response to influenza virus HA DNA following a single intramuscular administration indicated that a strong IFNγ secreting cellular response but a poor humoral response had been induced. Two booster immunisations were required to generate a significant virus-specific IgG response in the serum (Johnson et al., 2000). A subsequent study also reported that a single intramuscular immunisation could not induce antibody production (Kasinrerk et al., 2002).

When DNA vaccines for influenza virus were first studied, a construct encoding NP was shown to generate both humoral and cellular responses, as well as protection against lethal challenge. And it was suggested that protection was CTL mediated (Ulmer et al., 1993a). Further studies of DNA immunisation against influenza virus HA, as well as genes encoding internal proteins, and have analysed humoral as well as cellular immunity (Donnelly et al., 1995, Fu et al., 1997, Fynan et al., 1993, Kodihalli et al., 1999). In conjunction, a study

investigating protection following intramuscular NP DNA immunisation reported that depletion of $CD8^+$ T cells prior to a lethal challenge, completely abolished protection while depletion of $CD4^+$ cells had a partial effect (Ulmer et al., 1998).

In this study, characterisation of the immune response in challenged mice that had been prophylactically immunised demonstrated a significant influx of virus-specific T cells into the lungs. It was of interest to investigate whether this recruitment of virus-specific cells to the lungs afforded any protection from viral replication. It was observed that recruitment of effector $CD8^+$ T cells has the effect of reducing viral replication in the lungs to the extent that there is a 2 log reduction in viral titre in animals primed with pVac-NP before intranasal influenza virus challenge. Visually there were no obvious signs of pathology observed in these animals. The cellular influx did not completely abolish viral replication but resulted in a more rapid clearance of the virus from the lungs. *In vivo* depletion of the responding $CD8^+$ T cells was inconclusive and requires further investigation but appeared to have minimal effect on viral growth in the lungs. This data appears to conflict that of Ulmer *et al.* (1998). However, there is one major difference between the studies, and that is the dose of virus used for challenge. This study used a non-lethal challenge whereas Ulmer *et al.* (1998) used a lethal challenge and this could account for the abrogation of protection when $CD8^+$ T cells were depleted. The immunisation method should also be taken into consideration. This study used PMID or gene gun delivery of NP DNA whereas Ulmer *et al.* (1998) used intramuscular injection of plasmid. Gene gun immunisation is know to generate greater $CD8^+$ T cell responses (Trimble et al., 2003) which could also account for the differences seen between the studies.

To further complicate the discussion, a study focusing on the protection afforded by DNA vaccination of internal influenza virus proteins was recently carried out

(Epstein et al., 2000). The authors reported that intramuscular immunisation with plasmids expressing both influenza NP and matrix protein (M) provided protection from a lethal challenge. Depletion studies further characterised this response to be mediated by T cell immunity but $CD8^+$ T cells were not essential. It was observed that $CD4^+$ or $CD8^+$ T cells can promote survival and recovery from lethal influenza virus challenge in the absence of the other set.

Taken together, these data show that the depletion experiment in this study should be reinvestigated. Perhaps with a lethal challenge and definitely with the depletion of $CD4^+$ and $CD8^+$ T cell subsets both individually and together. This should aid in the clarification of reports published to date.

Many of the current studies on DNA vaccination are investigations concerned with generating the most robust immune response often following a reliable "prime boost" regimen. The studies reported here are only concerned with characterisation of the mucosal and systemic response to a single vaccination with DNA. Most encouragingly it has have demonstrated that epidermal PMID delivery of a plasmid encoding influenza virus NP DNA can induce a mucosal response in the D-NALT.

Chapter 5

Phenotypic Analysis of Influenza Virus Specific $CD8^+$ T Cells Distributed Throughout the Mucosal Immune System

5.1 Introduction

Lymphocyte migration from the blood into secondary lymphoid organs is mandatory for maintaining immune surveillance and providing efficient defense against invading pathogens. The major function of $CD8^+$ effector T cells is the killing of other cells, and direct contact with the target cell is required for this to happen. Target cells can be located anywhere in the body and thus effector $CD8^+$ T cells must demonstrate an ability to migrate throughout the body and effectively traffic to sites of inflammation. This migration is partly controlled by tissue-selective expression of endothelial adhesion molecules that bind ligands on the surface of circulating lymphocytes.

The aim of this study was to investigate the antiviral $CD8^+$ T cells generated by mice following experimental influenza virus infection or PMID of influenza virus NP DNA, with respect to their expression of adhesion molecules. Virus-specific $CD8^+$ T lymphocytes were assessed for their expression of the common adhesion/activation markers CD44, CD11a, CD62L and CD69. Subsequent *in vivo* transfer studies were used to analyse the migratory preferences of activated $CD8^+$ splenocytes. It is thought that the analysis of these cells could determine the efficacy of PMID immunisation at stimulating immune responses that migrate to similar sites to those observed in a protective response and help elucidate more information regarding the common mucosal immune system.

5.2 Results

5.2.1 Phenotypic Analysis of Influenza-Specific Effector $CD8^+$ T Cells

Single cell suspensions of cells isolated from various mucosal and systemic tissues were prepared and stained with influenza virus-specific H-2Db $NP_{366-374}$

tetramer as previously described. Cells were subsequently counterstained with anti-CD8 in combination with either anti-CD44, anti-CD11a, anti-CD62L or anti-CD69 before detection of immunofluorescence was carried out on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

Influenza virus-specific H-2Db $NP_{366-374}$ tetramers were used as a tool to identify virus specific cells generated by mice following intranasal influenza virus infection or PMID immunization using an influenza NP DNA plasmid. Identification of the influenza virus-specific cells allowed expression levels of a range of surface antigens implicated in adhesion and activation of leukocytes to be investigated.

CD44 is a cell adhesion receptor, and its principal ligand, hyaluronic acid, is a common component of extracellular matrices (Lesley et al., 1993). In the periphery, the level of expression of CD44 increases upon activation of B lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells (Budd et al., 1987, Hathcock et al., 1993). Memory cells can be recognised by their $CD44^{hi}$ phenotype (MacDonald et al., 1990). CD11a is the α_L chain of LFA-1 (CD11aCD18, $\alpha_L\beta_2$ integrin) a surface glycoprotein expressed on almost all leukocytes (Larson & Springer, 1990) and is involved in the multistep model of leukocyte recruitment. CD11a binds to its ligands ICAM-1 and ICAM-2 mediating the firm binding of rolling leukocytes to endothelium and promoting arrest and subsequent transmigration of leukocytes through the endothelial layer and into tissues (von Andrian et al., 1991). CD62L is a member of the selectin molecule family and is also known as L-selectin. It is expressed on thymocytes and peripheral leukocytes, including B and T cells (Iwabuchi et al., 1991). CD62L is required for lymphocyte homing to peripheral lymph nodes (Gallatin et al., 1983) and its expression, along with other markers, distinguishes naïve, effector and memory T cells (Sprent &

Tough, 1994). CD69 (very early activation marker) is closely related to NK cell activation molecules (Ziegler et al., 1994a) and is rapidly induced upon activation of lymphocytes (Ziegler et al., 1994b).

The characteristic expression patterns of the surface antigens CD44, CD11a, CD62L and CD69 on $CD8^+$ lymphocytes isolated from naïve animals were compared to that expressed by cells isolated from influenza virus infected mice and the results are shown in Figure 17. It was observed that $CD8^+CD44^{hi}$ and $CD8+CD11a^{hi}$ cells (upper right-hand quadrants) are more numerous in the spleens (and other tissues, data not shown) of mice infected with influenza virus. It was also observed that activated $CD8^+$ T cells expressing low levels of CD62L (upper left-hand quadrant) were also more numerous in the spleens of influenza infected animals. No difference in the level of expression of CD69 was observed between the mice.

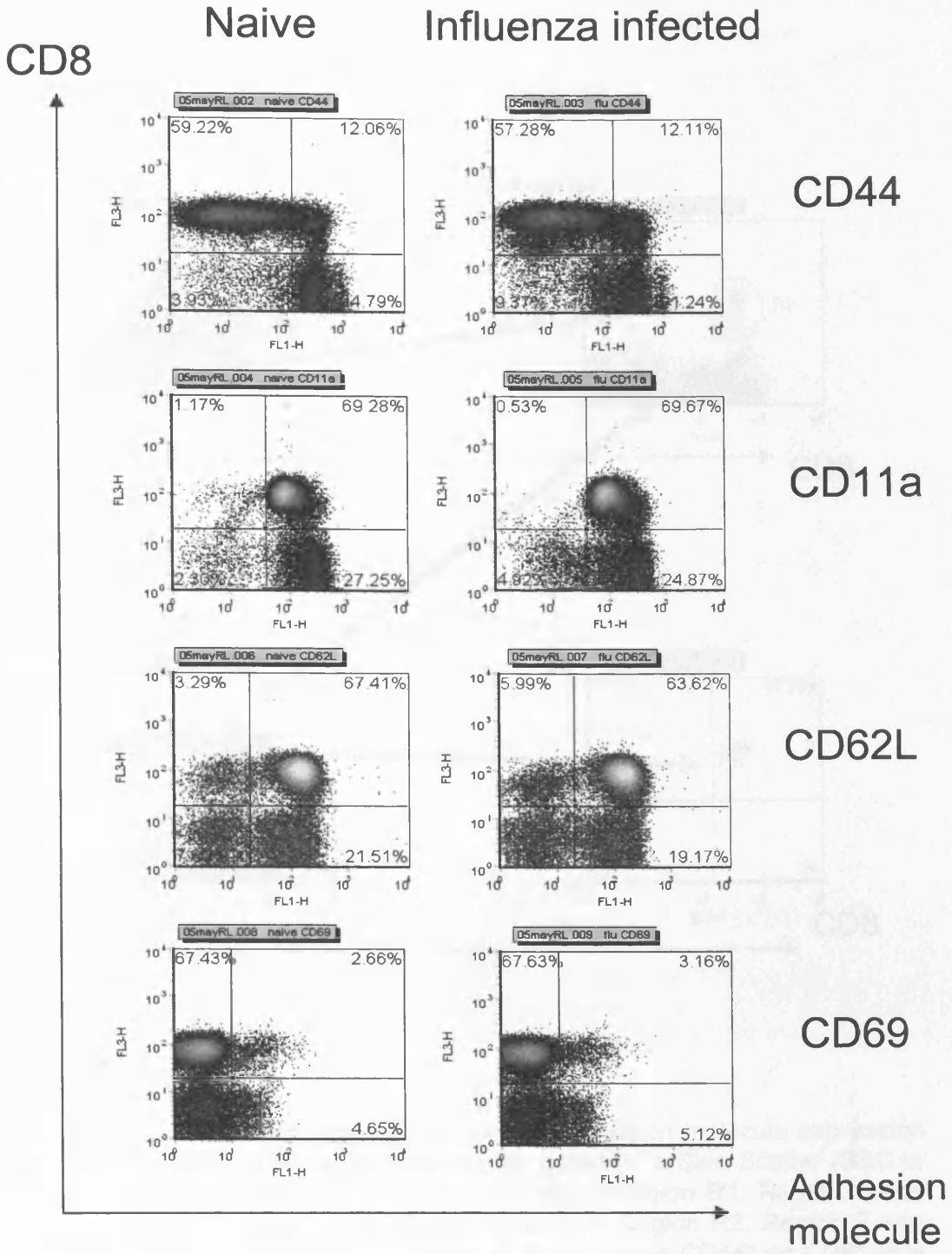


Figure 17: Adhesion molecule expression is similar in both naïve and influenza virus activated lymphocytes. C57BL/6 lymphocyte gated staining of CD8 enriched (negative selection) splenic lymphocytes which are either naïve or 10 days post influenza virus infection. The characteristic staining patterns of CD44, CD11a, CD62L and CD69 on total lymphocytes can be seen.

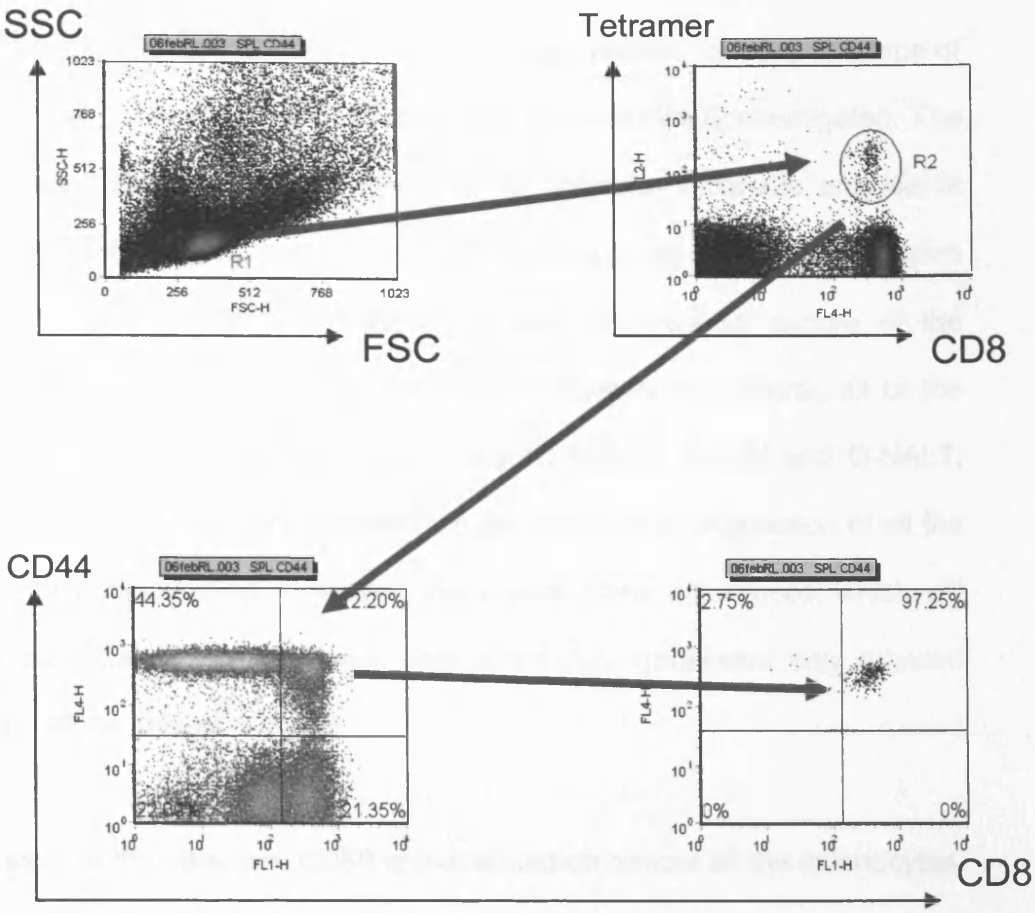


Figure 18: Shows the gating strategy adopted for adhesion molecule expression studies. Cell suspensions were first lymphocyte gated on a Side Scatter (SSC or granularity) v's Forward scatter (FSC or size) plot = Region R1. Region 1 was then assessed for expression of CD8 and tetramer = Region R2. Region 2 was then applied to adhesion molecule expression (in this case CD44) on $CD8^+$ cells (bottom left plot) to show the expression levels of adhesion molecule on $CD8^+$ $tetramer^+$ lymphocytes (bottom right plot).

At 10 days after the intranasal influenza virus infection of mice, the expression of the adhesion molecules on virus specific $CD8^+$ splenocytes was observed to be upregulated compared with the expression levels observed on $CD8^+$ T cells isolated from naive animals (Figure 17). It is likely that expression of various surface antigens may vary during the course of an infection, so the phenotype of cells isolated at 7, 10, 14, 21 and 35 days post-infection were investigated. The flow cytometry gating strategy adopted for all adhesion molecule analysis is shown in Figure 18. The adhesion molecule expression of splenic lymphocytes at various times post-infection (Figure 19) gives a universal picture of the phenotype of cells found in all the tissues investigated. In general, all of the tissues analysed, namely: lung, spleen, MedLN, MesLN, IngLN and D-NALT, expressed similar amounts and showed similar changes in expression of all the adhesion molecules studied. However, there were some differences, which will be addressed, but due to the large volume of data generated only relevant FACS plots will be shown.

At day 7, early in the infection, CD69 is expressed on almost all the splenocytes, and was also seen to be expressed at a similar level on the majority of lymphocytes found in the MedLN and in the lungs (data not shown). At this early timepoint (day 7) CD62L expression is high, indicating the relatively naïve phenotype of the virus-specific $CD8^+$ lymphocytes at this stage of infection. The level of CD11a expression in the spleen appeared to slightly increase and the other tissues examined showed a similar CD11a phenotype. The number of $CD8^+$ $tetramer^+$ cells showing a $CD44^{hi}$ phenotype varied during the course of infection. Early in the infection at day 7, CD44 expression was equally divided between $CD44^{lo}$ and $CD44^{hi}$. As the infection progressed, the $CD8^+$ $tetramer^+$ lymphocytes become predominantly $CD44^{hi}$ before expression was slightly down regulated. This was also observed to be the case for the other tissues examined

except within the IngLN where the majority of $CD8^+$ $tetramer^+$ cells remained $CD44^{lo}$. The D-NALT also showed some differences in staining, but this will be discussed in the next figure.

Figure 20 shows a snap shot of the adhesion molecule staining on cells isolated from various tissues at 14 days post influenza virus infection. This timepoint was chosen because it was the best representative timepoint and plots for all of the tissues could be shown. The $CD8^+$ $tetramer^+$ D-NALT population was observed to have an intermediate level of CD44 expression (Figure 20, outlined in red). CD44 expression in the D-NALT was observed to be at an intermediate level throughout the course of infection. Another significant result from this figure was the observation that CD69 is up regulated on the $CD8^+$ $tetramer^+$ lymphocytes isolated from the MedLN and the lungs (also indicated in red). A recent study has linked the expression of CD69, a marker of early activation, to the presence of antigen. Using the influenza mouse model, peptide stimulated proliferating $CD8^+$ ($CFSE^{low}$) cells were found to rapidly upregulate CD69 (Lawrence & Braciale, 2004). The authors suggested that undivided T cells and T cells early in their proliferative cycle may still have been in contact with antigen in the MedLN at day 5 of infection.

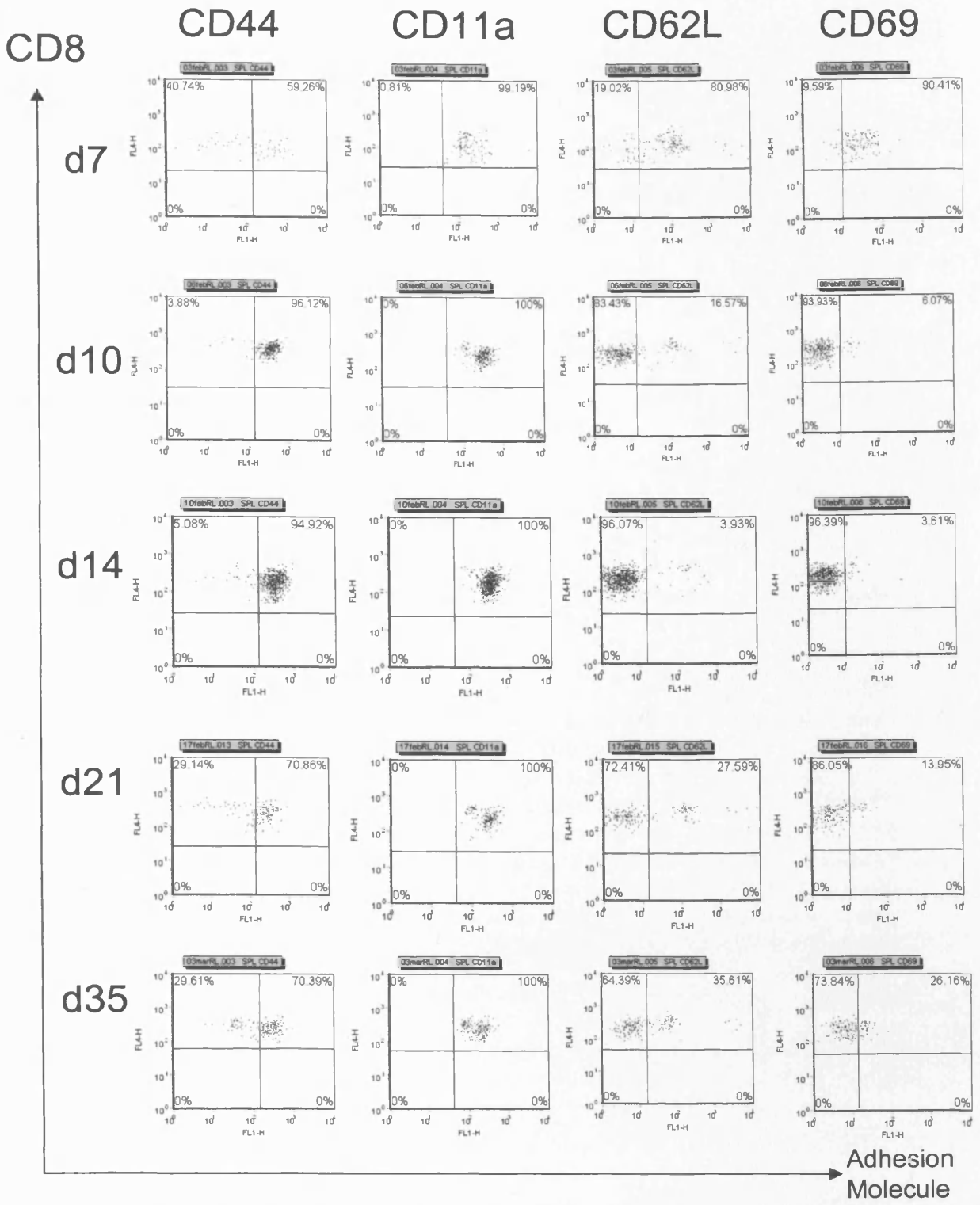
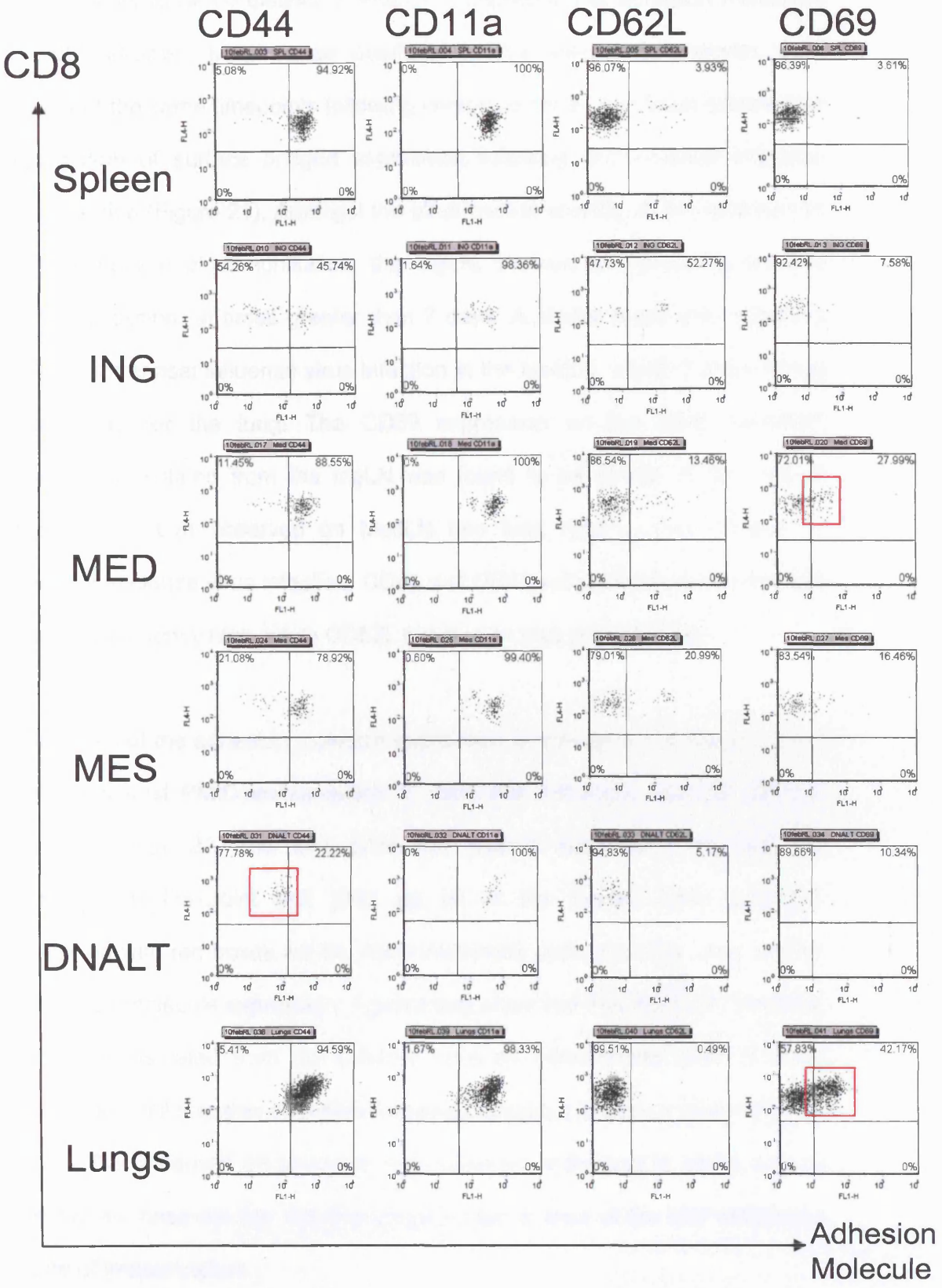


Figure 19: C57BL/6 mice were intranasally infected with 500 EID_{50} influenza virus A/PR/8/34 and tissues were collected for analysis of adhesion molecule expression on influenza virus-specific H-2Db $NP_{366-374}$ tetramer positive cells. This figure is a representative of the adhesion molecule staining on all tissues analysed and shows splenocytes at various time points following intranasal influenza virus infection. CD69 is found to be highly expressed early in the infection (d7), this was also found to be the case for lymphocytes isolated from the MedLN and lungs (data not shown). CD62L expression was also found to be high early in the infection indicating the naïve phenotype of the cells. Expression of CD44 was found to remain constant throughout the course of infection, while CD11a expression was observed to be slightly upregulated. These data are representative of two experiments and tissue pooled from five mice per timepoint.

Figure 20: C57BL/6 mice were intranasally infected with 500 EID_{50} influenza virus A/PR/8/34 and tissues were collected for analysis of adhesion molecule expression on influenza virus-specific H-2Db $NP_{366-374}$ tetramer positive cells. This figure shows the adhesion molecule staining on all tissues analysed at day 14 post influenza virus infection when the D-NALT populations were adequate enough to analyse. Lymphocytes isolated from the D-NALT at this and every timepoint showed a characteristic $CD44^{intermediate}$ phenotype. CD69 expression in the lungs and MedLN is found to be high suggesting that these cells are still in direct contact with antigen. These data are representative of two experiments and tissue pooled from five mice per timepoint.


CD44
CD11a
CD62L
CD69
CD8
Spleen
ING
MED
MES
DNALT
Lungs
Adhesion Molecule
5.08%
94.92%
0%
100%
96.07%
3.93%
96.39%
3.61%
54.26%
45.74%
1.64%
98.36%
47.73%
52.27%
92.42%
7.58%
11.45%
88.55%
0%
100%
86.54%
13.46%
72.01%
27.99%
21.08%
78.92%
0.60%
99.40%
79.01%
20.99%
83.54%
16.46%
77.78%
22.22%
0%
100%
94.83%
5.17%
89.66%
10.34%
5.41%
94.59%
1.67%
98.33%
99.51%
0.49%
57.83%
42.17%

At various timepoints following PMID immunisation of influenza virus NP DNA, there appears to be no distinct difference in expression of adhesion molecules by $CD8^+$ $tetramer^+$ lymphocytes isolated from the spleen. Splenocytes were isolated at the same timepoints following immunisation as had been selected for investigation of surface antigen expression following experimental influenza virus infection (Figure 21). Amongst the other tissues examined, the lymph node draining the site of immunisation, the IngLN, showed an increasing level of CD69 expression, at times greater than 7 days. A similar result was observed following intranasal influenza virus infection in the MedLN, which is the draining lymph node for the lung. The CD69 expression on the $CD8^+$ $tetramer^+$ lymphocytes isolated from the IngLN was found to be similar to, but not as extensive as, that observed on MedLN and lung lymphocytes induced by intranasal influenza virus infection. CD44 and CD11a expression were observed to be predominantly high, while CD62L expression was primarily low.

A snap shot of the adhesion molecule expression across all of the tissues tested at 10 days post PMID immunisation of mice with influenza virus NP DNA is shown in Figure 22. This time point was chosen because it was the best representative timepoint and plots for all of the tissues were available. Highlighted with red boxes are the most interesting plots from this cross section of adhesion molecule expression. Again it was observed that the $CD8^+$ $tetramer^+$ lymphocytes isolated from the D-NALT have an intermediate level of CD44 expression, similar to that observed following intranasal influenza virus infection. $CD69^{hi}$ $CD8^+$ $tetramer^+$ lymphocytes were observed in the IngLN, which may be expected as these are the draining lymph nodes of area of the skin which was the site of immunisation.

One final observation was the response seen in the lungs. Following the discovery that two gene gun immunisations one week apart has the effect of boosting the specific $CD8^+$ response (personal communication with Dr Fiona Cook) this technique was used to boost the numbers of tetramer positive cells in tissues like the D-NALT where very few cells can be isolated. Previously, with ELISPOT data (Figure 13), when only one immunisation was given, there was a very limited response observed in the lungs. Unfortunately due to time constraints, ELISPOT characterisation of the cellular response to gene gun immunisation with two immunisations could not be investigated after this double immunisation regimen had been used.

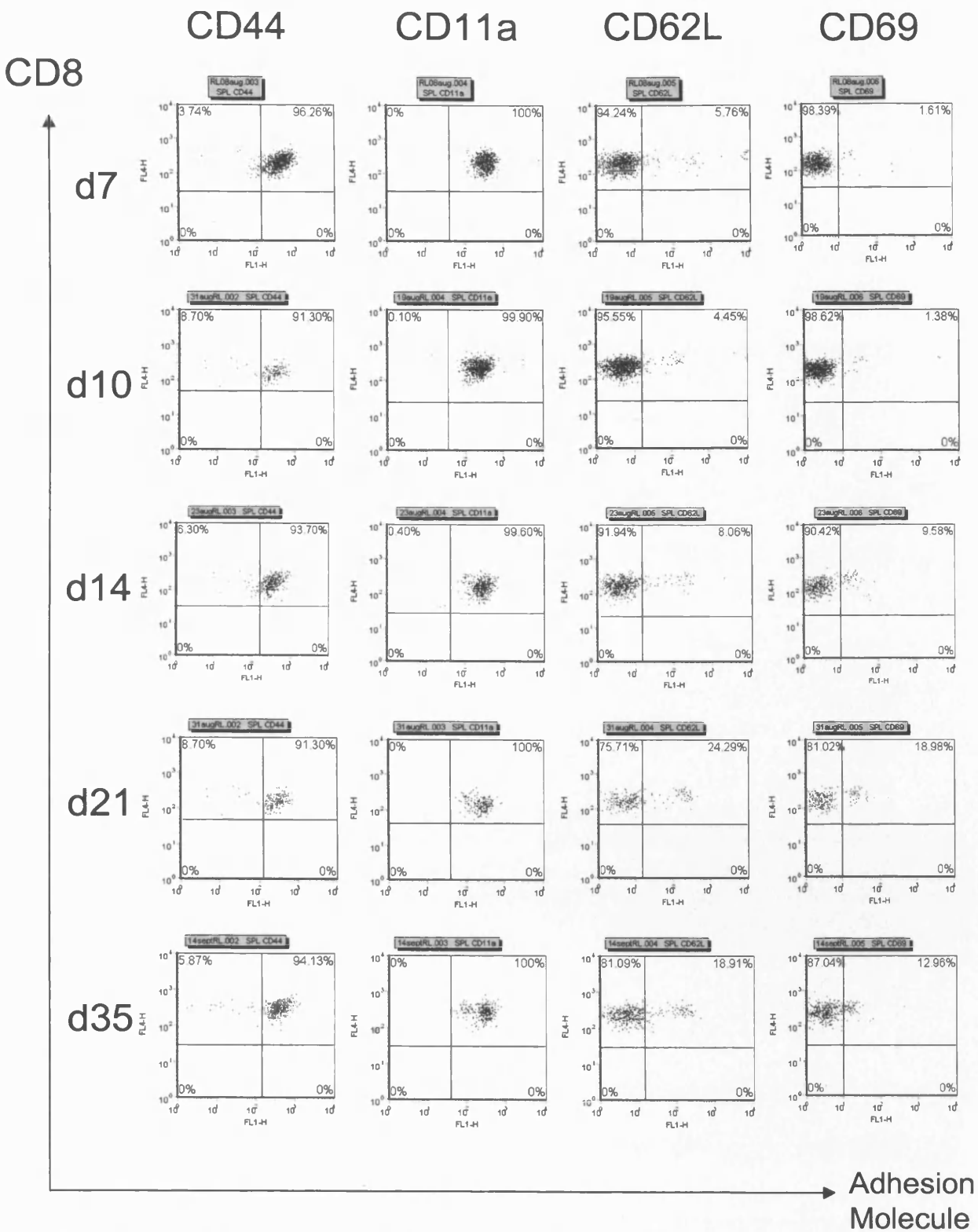
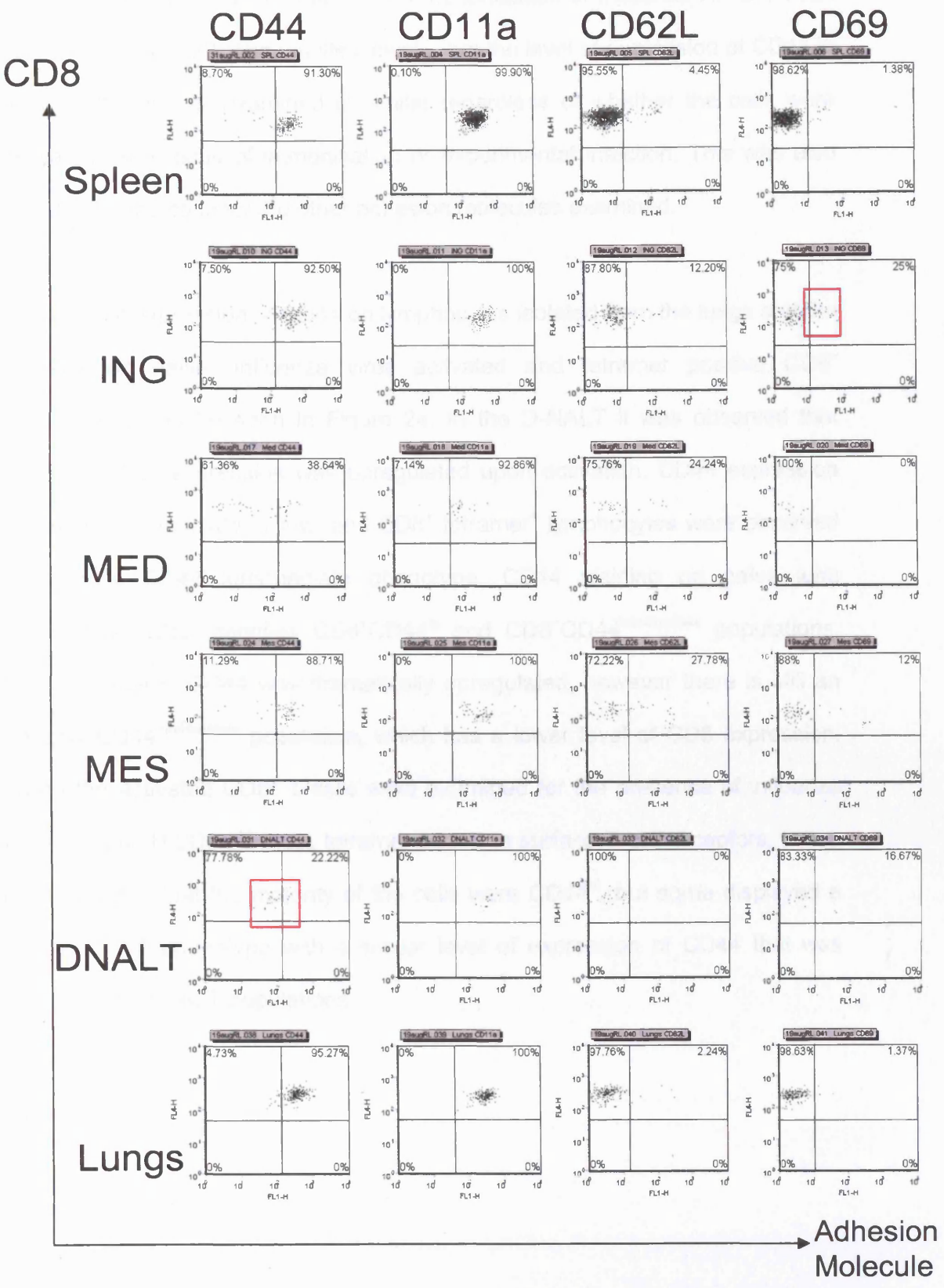


Figure 21: C57BL/6 mice were immunised twice, one week apart by PMID of influenza virus NP DNA. Each immunisation comprised 2 non-overlapping doses of approximately 0.5μg DNA. Tissues were collected for analysis of adhesion molecule staining of virus-specific H-2Db $NP_{366-374}$ tetramer positive cells. This figure is a representative of the adhesion molecule staining on all tissues analysed and shows splenocytes at various time points following PMID immunisation. No major difference in adhesion molecule expression was observed up to day 35 post immunisation except that of CD69 which was found to be upregulated over the timecourse in the spleen and more markedly in the IngLN. These data are representative of two experiments and tissue pooled from five mice per timepoint.

Figure 22: C57BL/6 mice were immunised twice, one week apart by PMID of influenza virus NP DNA. Each immunisation comprised 2 non-overlapping doses of approximately 0.5μg DNA each. Tissues were collected for analysis of adhesion molecule staining of virus-specific H-2Db $NP_{366-374}$ tetramer positive cells. This figure shows the adhesion molecule staining on all tissues analysed at day 10 post PMID immunisation when the D-NALT populations were adequate enough to analyse. Lymphocytes isolated from the D-NALT at this and every timepoint showed a characteristic $CD44^{intermediate}$ phenotype. CD69 expression in the IngLN is found to be high suggesting that these cells are still in direct contact with antigen. These data are representative of two experiments and tissue pooled from five mice per timepoint.


CD44
CD11a
CD62L
CD69
CD8
Spleen
ING
MED
MES
DNALT
Lungs
Adhesion Molecule

The expression of CD44 in the various tissues examined following both intranasal influenza infection and PMID immunisation of influenza NP DNA can be seen in Figure 23. The results indicate that the level of expression of CD44 in each of the tissues examined is similar regardless of whether the cells were activated as a result of immunisation or experimental infection. This was also found to be the case for the other adhesion molecules examined.

The level of expression of CD44 on lymphocytes isolated from the lungs and the D-NALT on naïve, influenza virus activated and tetramer positive $CD8^+$ lymphocytes can be seen in Figure 24. In the D-NALT it was observed that although $CD8^+$ expression was upregulated upon activation, CD44 expression remained intermediate or low, and $CD8^+$ $tetramer^+$ lymphocytes were observed to have a CD44 intermediate phenotype. CD44 staining on naïve lung lymphocytes also identifies $CD8^+CD44^{lo}$ and $CD8^+CD44^{intermediate}$ populations. Upon activation, CD44 was dramatically upregulated, however there is still an obvious $CD44^{intermediate}$ population, which has a lower level of CD8 expression. When the activated $CD8^+$ T cells were examined for the presence of influenza virus-specific H-2Db $NP_{366-374}$ tetramer bound to surface T cell receptors, it was observed that that the majority of the cells were $CD44^{hi}$, but some displayed a $CD44^{intermediate}$ phenotype with a similar level of expression of CD44 that was observed in D-NALT populations.

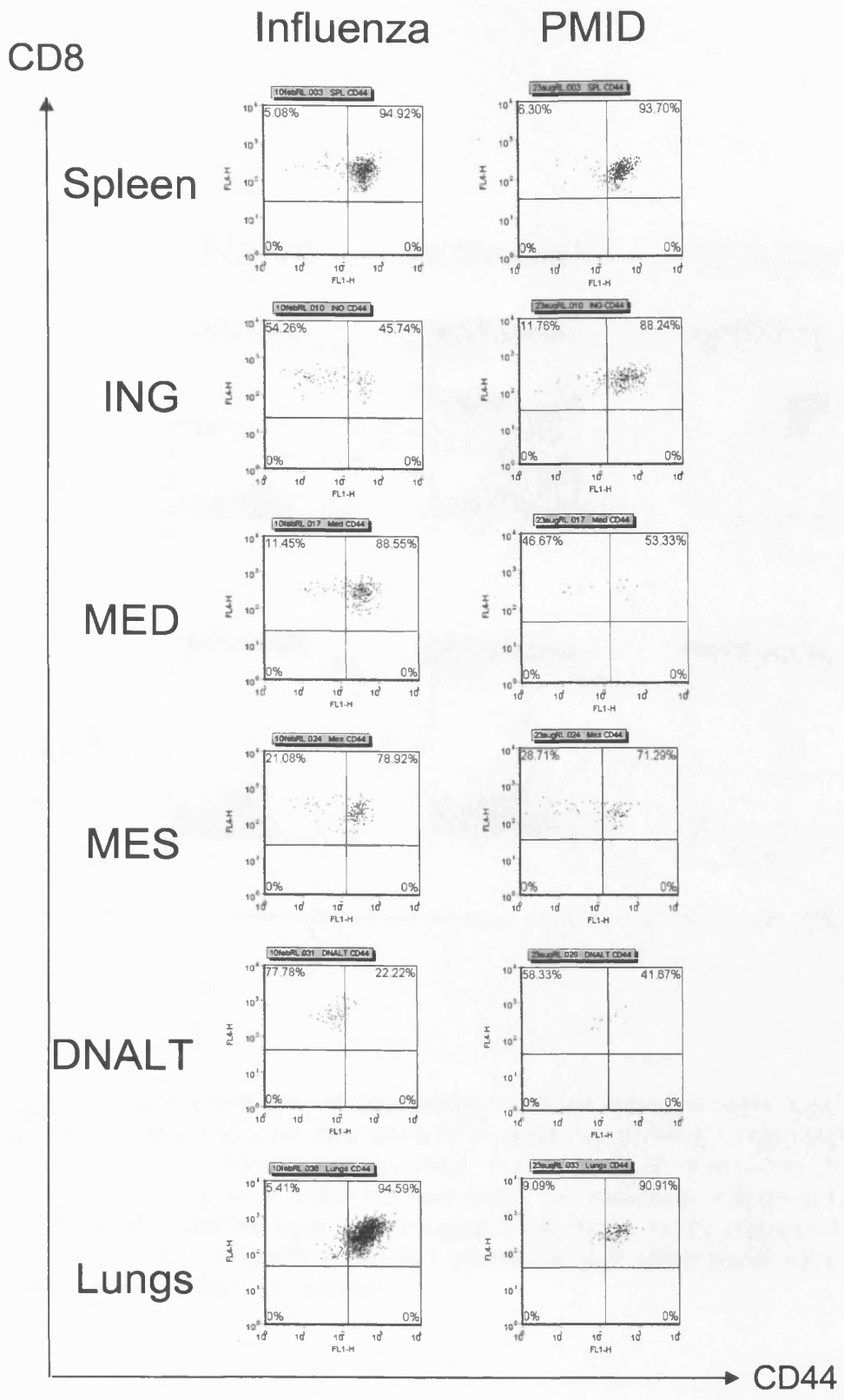


Figure 23: a comparison of the CD44 staining of tetramer positive cells at day 14 following activation by either intranasal influenza virus A/PR/8/34 infection or two PMID immunisations, one week apart, comprising two non-overlapping doses of 0.5μg influenza NP DNA. The levels of expression of CD44, and the other adhesion molecules (data not shown) were observed to be similar irrespective of whether cells had been activated by immunisation or experimental infection.

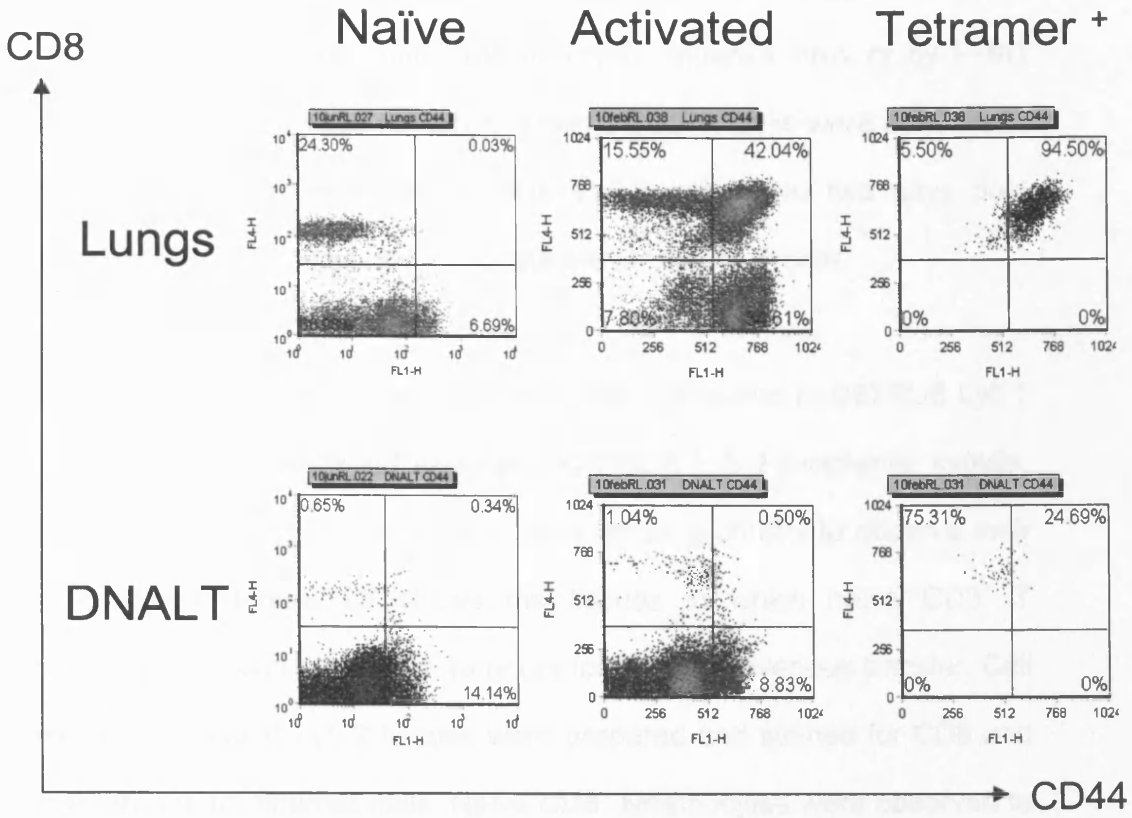


Figure 24: Shows the similarity in expression of CD44 between naïve lung and D-NALT lymphocytes and how they were both observed to have a population of CD44 intermediate expressing lymphocytes. It can be seen that CD44 is not upregulated in the D-NALT following activation by intranasal influenza virus infection. Tetramer positive lung lymphocytes were found to be predominantly $CD44^{hi}$ while the tetramer positive D-NALT population was observed to have and intermediate level of CD44 expression.

5.2.2 Virus Specific Lymphocytes Generated by PMID Immunisation Home Preferentially to Respiratory Mucosal Sites.

The homing properties of naïve and effector $CD8^+$ T lymphocytes from C57BL/6 Ly5.1 mice were investigated in an adoptive transfer model. Effector $CD8^+$ cells were generated either by intranasal infection with influenza virus or by PMID immunisation of influenza virus NP DNA. Splenic $CD8^+$ T cells were transferred intravenously into naïve recipient C57BL/6 Ly5.2 mice, and two days post transfer, tetramer staining was used to locate effector $CD8^+$ T cells.

Experiments were designed to follow effector cells generated in C57BL/6 Ly5.1 mice once they were transferred into naive C57BL/6 Ly5.2 recipients. Initially, naïve splenic $CD8^+$ lymphocytes were transferred as a control to observe their homing properties. Figure 25 shows the tissues in which naïve $CD8^+$ T lymphocytes are found to migrate to 48 hours following intravenous transfer. Cell suspensions of C57BL/6 Ly5.2 tissues were prepared and stained for CD8 and Ly5.1 to locate the transferred cells. Naïve $CD8^+$ lymphocytes were observed to be circulating in the blood and throughout the peripheral lymph nodes. They were also found in the lungs, liver and spleen. Most significantly, no naïve $CD8^+$ lymphocytes were detected in the D-NALT where only background staining was observed.

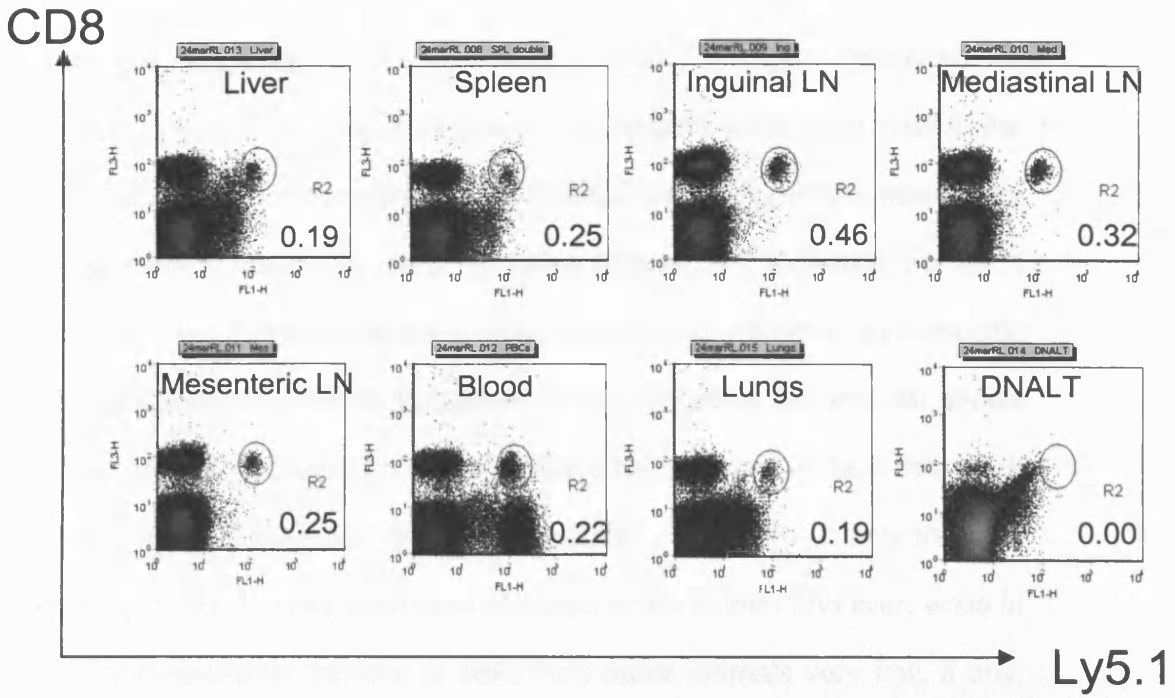


Figure 25: Total naïve CD8⁺ T lymphocytes were MoFlo sorted from the spleens of 20 C57BL/6 Ly5.1⁺ mice and transferred intravenously into naïve C57BL/6 Ly5.2⁺ recipients. 48 hours post transfer, the location of naïve splenic C57BL/6 Ly5.1⁺ CD8⁺ lymphocytes was assessed by flow cytometry. Region R2 indicates the Ly5.1⁺ CD8⁺ transferred cells which were predominantly found in the peripheral lymph nodes, spleen and circulating in the blood. Only background staining was observed in the D-NALT. The percentage of total cells that is R2 is shown on each plot. These data are representative of one experiment.

In the next experiment, naïve splenic $CD8^+$ T cells were replaced with total splenic $CD8^+$ T cells isolated 12 days post intranasal influenza virus infection. The location of the cells transferred from the influenza virus infected mice is shown in Figure 26. Transferred populations of total CD8 cells were found in all tissues including the lungs and D-NALT (Figure 26a). This figure clearly shows the trafficking of total $CD8^+$ T cells (region R2) activated by influenza virus to the D-NALT, which should be compared to the D-NALT plot in Figure 25, where only background staining was observed when naïve cells were transferred. For each tissue, region 2 was further analysed for the presence of influenza virus-specific H-2Db $NP_{366-374}$ tetramer bound to surface T cell receptors. Figure 26b shows the tetramer staining of $CD8^+Ly5.1^+$ lymphocytes found in the various tissues. It can be seen that the influenza virus-specific $CD8^+$ cells preferentially traffic to the lungs and D-NALT. They were also detected in the spleen and liver, while in contrast to the results for transfer of cells from naïve animals very few, if any, were observed to be circulating in the blood or to traffic to the lymph nodes.

Finally, the transfer was repeated with total $CD8^+$ splenic T cells isolated from mice following PMID immunisation of NP DNA (Figure 27). Similar results were observed to those following the transfer of cells activated by intranasal influenza virus infection. Again, it can be seen that activation leads to the trafficking of $CD8^+Ly5.1^+$ transferred cells to the D-NALT (Figure 27a). PMID activation appears to favour migration to the D-NALT where 19% of the transferred cells were found to be tetramer positive.

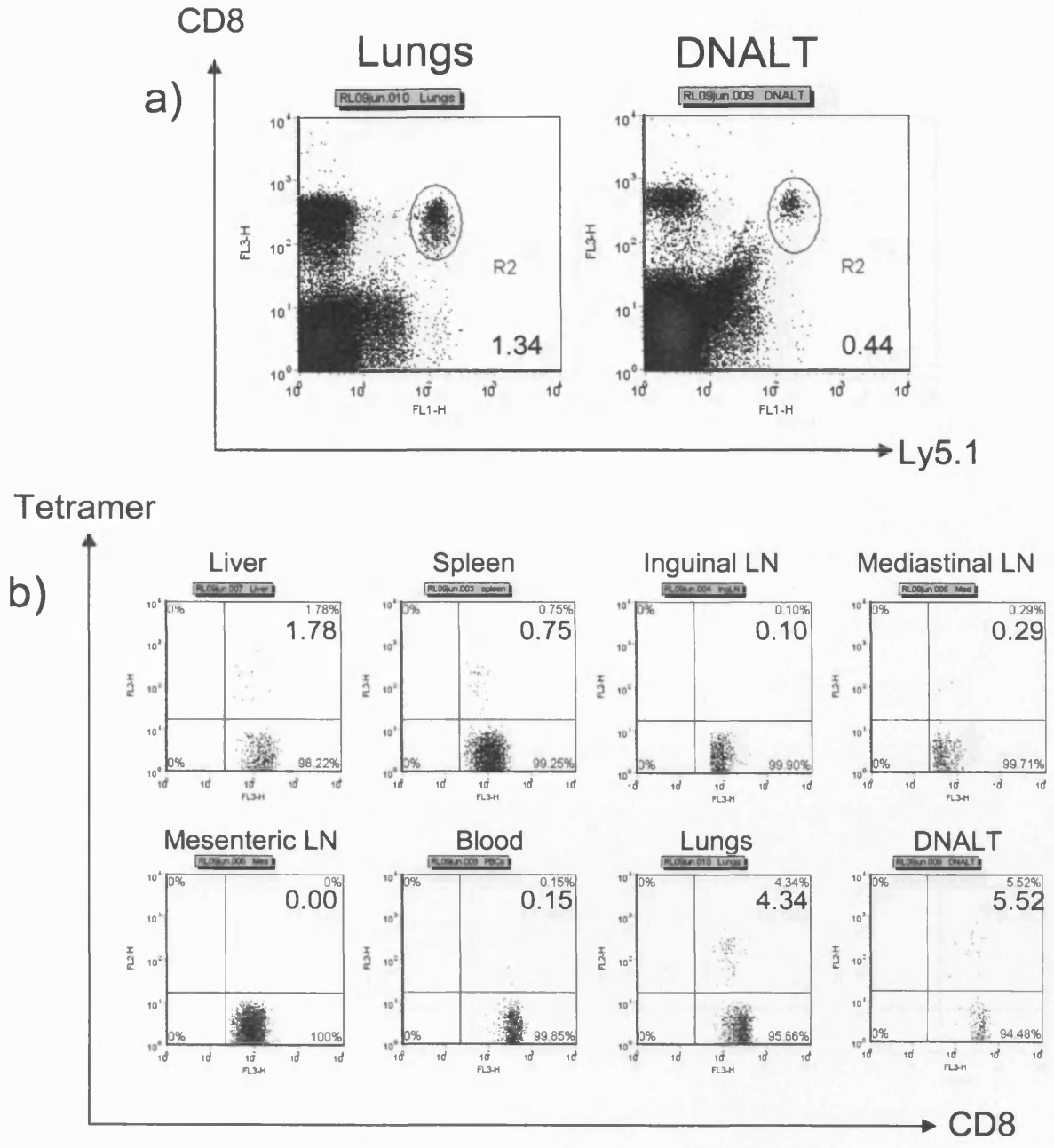


Figure 26: Total CD8⁺ T lymphocytes were MoFlo sorted from the spleens of 20 C57BL/6 Ly5.1⁺ mice that had been infected with 500 EID_{50} influenza virus A/PR8/34 virus 12 days previously. The cells were transferred intravenously into naïve C57BL/6 Ly5.2⁺ recipients. 48 hours post transfer, the location of splenic Ly5.1⁺ CD8⁺ lymphocytes was assessed by flow cytometry a) splenic Ly5.1⁺ CD8⁺ lymphocytes (R2) in the lungs and D-NALT demonstrates the trafficking of activated CD8⁺ T cells to the D-NALT b) indicates the presence of influenza virus-specific H-2Db $NP_{366-374}$ tetramer bound to surface T cell receptors of region R2 lymphocytes. Ly5.1⁺CD8⁺Tetramer⁺ transferred cells were predominantly observed in the lungs and D-NALT, but to some extent in the liver and spleen. Unlike naïve T cells, very little if any trafficking was observed to the lymph nodes. The percentage of total Ly5.1⁺CD8⁺ cells found in each tissue that was tetramer positive is shown on each plot. These data are representative of one experiment.

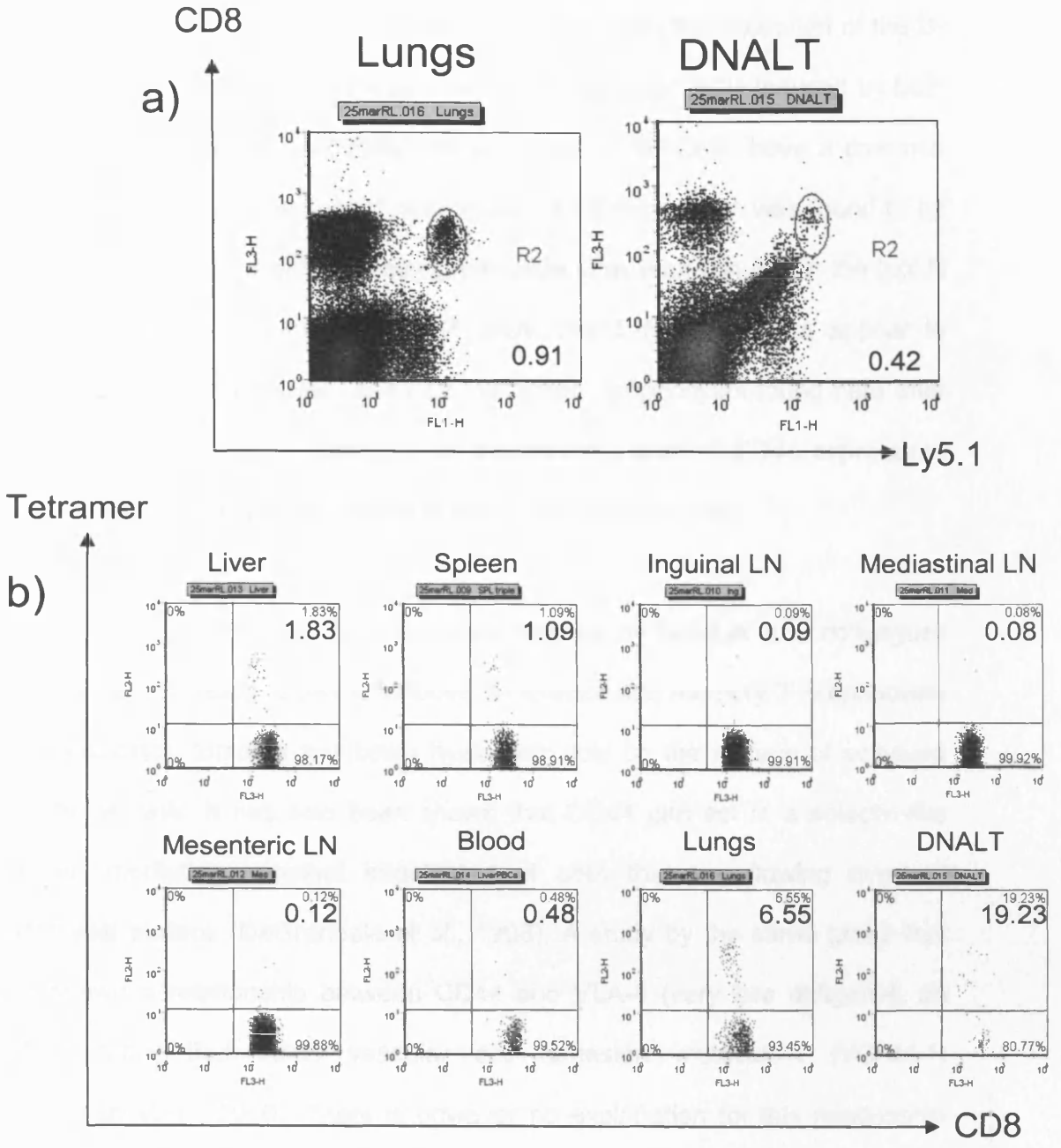


Figure 27: Total CD8⁺ T lymphocytes were MoFlo sorted from the spleens of 20 C57BL/6 Ly5.1⁺ mice immunised twice, one week apart with 2 non overlapping doses of 0.5μg influenza virus NP DNA, and transferred intravenously into naïve C57BL/6 Ly5.2⁺ recipients. 48 hours post transfer, the location of splenic Ly5.1⁺ CD8⁺ lymphocytes was assessed by flow cytometry a) splenic Ly5.1⁺ CD8⁺ lymphocytes (R2) in the lungs and D-NALT demonstrates the trafficking of activated CD8⁺ T cells to the D-NALT b) indicates the presence of influenza virus-specific H-2Db $NP_{366-374}$ tetramer bound to surface T cell receptors of region R2 lymphocytes. Ly5.1⁺CD8⁺Tetramer⁺ transferred cells were predominantly observed in the lungs and D-NALT, but to some extent in the liver and spleen. Unlike naïve T cells, very little if any trafficking was observed to the lymph nodes. The percentage of total Ly5.1⁺CD8⁺ cells found in each tissue that was tetramer positive is shown on each plot. These data are representative of one experiment.

5.3 Discussion

In the first part of this study it has been shown that with the exception of the D-NALT, regardless of the tissue examined, $CD8^+$ $tetramer^+$ cells induced by both influenza virus infection and PMID immunisation of NP DNA, have a common $CD44^{hi}CD11a^{hi}CD62L^{lo}$ activated phenotype. CD69 expression was found to be high in the lungs and MedLN following influenza virus infection and in the IngLN following PMID of influenza virus NP DNA. The D-NALT did not appear to upregulate CD44 expression, and $CD8^+$ $tetramer^+$ lymphocytes found here after infection or immunisation displayed an intermediate level of CD44 expression, similar to that found on a population in naïve lung lymphocytes.

CD44 was originally linked to lymphocyte homing by Butcher and colleagues (Jalkanen et al., 1987). CD44 is induced on effector and memory T lymphocytes and metastastic tumours and binds hyaluronic acid on the surface of activated endothelial cells. It has also been shown that CD44 can act in a selectin-like manner, mediating transient attachment of cells that are flowing over an endothelial surface (DeGrendele et al., 1996). A study by the same group has also shown a relationship between CD44 and VLA-4 (very late antigen-4, an $\alpha_4\beta_1$ integrin) that binds vascular cell adhesion molecule-1 (VCAM-1) (Siegelman et al., 2000). There is however no explanation for this relationship from which other integrins, like LFA-1, are excluded. It was also shown that absence of the cytoplasmic domain of CD44 abrogates this association with VLA-4 and resultant firm adhesion. Furthermore, CD44 can be immunoprecipitated with VLA-4 but not LFA-1 and this association requires the cytoplasmic domain of CD44. CD44 lacking the cytoplasmic domain is unable to associate with VLA-4 to produce firm adhesion both *in vitro* and *in vivo* (Nandi et al., 2004).

These data suggest that the combination of CD44/hyaluronic acid and VLA-4/VCAM-1 act together to aid entry of effector T lymphocytes into sites of inflammation and may suggest that earlier results showing that VLA-4 can directly mediate tethering and rolling (Alon et al., 1995) may have to be reconsidered. It could be possible that the studies demonstrating VLA-4 tethering to activated endothelial cells could be as a result of its close association with CD44.

Several studies have shown CD44 to act as a pro-inflammatory receptor in a variety of murine models including colitis, arthritis and hypersensitivity responses (Camp et al., 1993, Mikecz et al., 1995, Wittig et al., 2000). More recently, CD44 has also been reported to play a role in coordinating allergic airway inflammation. Monoclonal antibodies were shown to neutralise or cause shedding of CD44, thus blocking hyaluronic acid binding and reducing allergic respiratory inflammation by preventing lymphocyte and eosinophil accumulation in the lung (Katoh et al., 2003). By contrast, results from another study have demonstrated a protective anti-inflammatory role for CD44 in the lung (Teder et al., 2002). Here, $CD44^{-/-}$ mice developed inflammation following non-infectious lung injury which could be partially reversed following reconstitution with $CD44^{+}$ cells. However, using monoclonal antibodies, Katoh *et al.* did not deplete CD44 expressing alveolar macrophages which are likely to be critical in the resolution of lung inflammation, and this may explain the differences seen.

One final study on CD44 worth mentioning was carried out in a model of *Mycobacterium bovis* infection which examined the expression of CD44 on activated bovine T cells. This study showed that CD44 expression on bovine T cells, obtained from the lungs of infected cattle, was down regulated compared to that of naïve control animals (Waters et al., 2003). In a mouse model of

rheumatoid arthiritis, stimulation of CD44 by binding to fragmented hyaluronic acid was shown to upregulate Fas expression, enhancing Fas-mediated apoptosis of CD44 expressing synovial cells (Fujii et al., 2001). With this finding the authors suggest that there could be selective reduction of $CD44^{hi}$ cells mediated by apoptosis.

Taking this information into consideration, it would be interesting to look at the expression of the $\alpha_4\beta_1$ integrin VLA-4 on $CD8^+$ $tetramer^+$ lymphocytes found in the D-NALT following both infection and immunisation and to compare it to the other tissues examined. If CD44 does have a close relationship to VLA-4 then this may be reflected in the level of VLA-4 expression in the D-NALT.

Considering the reports of CD44 as a pro-inflammatory receptor, the intermediate level of CD44 expression on $CD8^+$ $tetramer^+$ lymphocytes found in the D-NALT may be explained by the observation that the D-NALT is a site where little inflammation is observed, and viral clearance is delayed in comparison to the lung following intranasal influenza virus infection (Dr S. Hou, unpublished data). A reduced level of CD44 expression on NALT lymphocytes could perhaps be a characteristic that aids in the control of and the accumulation of inflammatory cells as was observed by Teder *et al.* The D-NALT may also contain a unique environment which requires the downregulation of CD44 to maintain these cells or allow higher throughput of cells through the tissue.

A recent study investigating the phenotype of activated virus-specific $CD8^+$ T cells following influenza virus infection examined the expression of LFA-1 α-chain (CD11a) and VLA-4 (CD49d). Purified naïve $CD8^+$ T cells were used in an adoptive transfer experiment where naïve T cells were transferred intravenously before an influenza virus challenge (Lawrence & Braciale, 2004). The authors

report low levels of expression of both markers on the naïve undivided transferred cells which was dramatically upregulated when the cells were activated by viral infection. A similar result was observed in this study with CD11a expression, however, CD49d expression was not assessed. As previously discussed, these integrins are known to be important for the interaction of activated T cells with endothelium at sites of inflammation and they have been directly implicated in the recruitment of T cells to the lungs (Feng et al., 2000, Thatte et al., 2003, Wolber et al., 1998).

Attempts were made to phenotype influenza virus-specific $CD8^+$ T cells with regard to their expression of the $\alpha_4\beta_7$ integrin (LPAM-1). This was however unsuccessful due to technical difficulties. ProImmune, the commercial source for multimer stocks, stopped production of tetramer. Some of the new ProImmune Pentamer was tested but results were disappointing as pentamer positive populations were very dim and it proved hard to isolate positive populations, especially when there were very few virus specific $CD8^+$ T cells in the cell suspension.

CD69 upregulation on T cells is linked to TCR engagement by antigen (Cosulich et al., 1987, Ziegler et al., 1994a). When antigenic stimulation is withdrawn, CD69 expression subsequently declines (Testi et al., 1994). A study by Lawrence and Braciale (2004) used the influenza mouse model and linked CD69 expression with proliferating cells ($CFSE^{low}$) in the MedLN at 5 days post-infection. This was assumed to result from continued antigenic stimulation. This is understandable at an early timepoint post infection, but at day 14 (and beyond) the data generated as part of this thesis shows $CD69^{hi}$ cells present in the lungs and MedLN. It is likely that virus is cleared by this timepoint

(Eichelberger *et al.* (1991) and Figure 15) therefore CD69 expression is likely to be as a result of continued cellular activation at these sites.

Following the phenotypic analysis of $CD8^+$ $tetramer^+$ cells found following infection and immunisation, this study moved to characterise these cells further by investigating their homing properties. It was hoped that this would reveal whether the immune response generated by PMID immunisation of NP DNA was a good as that induced by experimental infection. Using an adoptive transfer model, the characteristic homing pattern of naïve $CD8^+$ T cells was compared to the antigen specific effector $CD8^+$ T cells generated by influenza virus infection or immunisation. As expected, naïve T cells were found to be predominantly circulating through the blood and lymph nodes. Upon activation however, the homing properties of T cells was dramatically altered. Activated T cells showed preferential localisation to non lymphoid tissues, which has previously been shown to occur independently of the infectious agent, infection route and tissue of origin (Masopust et al., 2001, Masopust et al., 2004). This study has subsequently shown the previously undocumented preferential migration of antigen specific effector $CD8^+$ T cells to the D-NALT.

In an early experiment in the field of lymphocyte homing studies, Gowans and Knight (1964) transferred thoracic duct lymphocytes intravenously into naïve recipients and detected donor lymphocytes in the recipient thoracic duct lymph. This experiment demonstrated that lymphocytes continuously recirculate between the blood and lymph. The authors also demonstrated that small lymphocytes cross high endothelial venules to enter lymph nodes. Small lymphocytes (which would be primarily made up of naïve T cells but would contain some circulating memory cells) were detected in the lymph nodes, white pulp of the spleen and PP. The number of small lymphocytes detected in the

other tissues was negligible providing an early suggestion that naïve lymphocytes may not enter non-lymphoid tissues. Experiments have demonstrated that naïve lymphocytes rarely enter non-lymphoid tissue. This has been shown using *in vivo* migration studies and adoptive transfer of traceable naïve $CD8^+$ T cells, which did not enter the intestinal lamina propria or epithelium unless activated (Kim et al., 1997). This study demonstrated limited trafficking of naïve lymphocytes to non-lymphoid tissues including the lung and liver (Figure 25). This observation is supported by data reported by Westermann and colleagues who recorded limited trafficking of naïve T cells through the skin and liver (Westermann & Pabst, 1996).

When naïve T cells encounter antigen in a lymph node, their homing pattern is dramatically changed. Primary infection with a variety of viruses has been associated with the appearance of activated antigen-specific $CD8^+$ T cells in non-lymphoid tissues. For example, following infection with LCMV or influenza virus, antigen-specific $CD8^+$ T cells have been detected in bone marrow (Marshall et al., 2001, Slifka et al., 1997). Analysis of primary immune responses to infection with influenza virus (Flynn et al., 1998) or Sendai virus (Usherwood et al., 1999a) has shown substantial numbers of antigen specific $CD8^+$ T cells in the lung, which are likely to have migrated to that site after primary activation in the draining lymph node (Lawrence & Braciale, 2004). Belz *et al.* (1998) have also shown the presence of antigen-specific $CD8^+$ T cells in the liver following influenza virus infection (Belz et al., 1998) which was not investigated in this study, but data does show the preference of activated $CD8^+$ T cells to migrate to the liver. This is of interest considering that productive infection of influenza virus is restricted to lung epithelial cells (Eichelberger et al., 1991b). The liver has also recently been shown to be a site of specific $CD8^+$ T cells following DNA vaccination with plasmid expressing hepatitis B surface antigen (Dikopoulos et

al., 2003). More recently, it has been shown that preferential migration of effector $CD8^+$ T cells, induced both by viral and bacterial infection, is to non-lymphoid tissues (Masopust et al., 2004). Together, these data demonstrate that activated T cells migrate to sites of infection and traffic to other, potentially uninfected tissues.

Data recorded in this study demonstrates that influenza virus activated and PMID activated virus specific $CD8^+$ T cells, are effectively identical with regard to expression of the adhesion molecules examined. However, those cells activated by PMID immunisation of influenza virus NP DNA appear to have a preference to migrate to the D-NALT, the mechanism of which remains to be elucidated. It is possible that the preferential migration of activated T cells to the D-NALT could be specific to DNA immunisation using a ballistic device and it would be interesting to repeat the transfer study with $CD8^+$ T cells activated by intramuscular DNA immunisation to investigate this further.

The homing of effector/activated $CD8^+$ T cells to the D-NALT and lungs observed with the adoptive transfers from influenza virus infected spleens is not surprising considering that these cells are perhaps homing to the sites of first stimulation/activation. However, the homing of effector/activated $CD8^+$ T cells to the same tissues after PMID implies that these tissues are perhaps sites where effector/memory T cells actively home to, perhaps in their role of immune surveillance.

Chapter 6

Discussion

The main aim of this study was to characterise the mucosal and systemic immune responses in a murine model of experimental influenza virus infection and to compare the results with the immune responses elicited following vaccination. Influenza virus infection can generate sterilising immunity and it was hoped that by defining the immune responses that contribute to this protection, a useful comparison for the assessment of potential efficacy of a DNA vaccine candidate could be made. It was also of interest to assess whether DNA vaccination by PMID could elicit a mucosal immune response.

The initial study was designed to characterise the humoral and cellular immune responses generated by experimental intranasal influenza virus infection of mice. ELISA data showed that potential correlates of protection were a dominant virus-specific Ig response in the MedLN, spleen and to some extent the lungs and D-NALT. Characterisation of the cellular response showed that a dominant virus-specific $CD8^+$ IFNγ secreting cellular response in the lungs correlated with protection. Recent studies on the induction of immune responses following influenza virus infection have shown that the response is initiated in the MedLN and the virus-specific effector $CD8^+$ T cells generated there go on to seed populations in the lung and spleen (Lawrence & Braciale, 2004). If a novel vaccine is to mimic influenza virus infection, it is likely that initiation of a cellular immune response in the MedLN is likely to contribute to protective efficacy.

Surprisingly, virus-specific $CD8^+$ IFNγ secreting cells were also observed in the MesLN. This leads to the question of whether activated virus-specific lymphocytes traffic to all tissues during and/or after infection. Such a strategy could be justified by the immune system to ensure that an invading pathogen is completely eliminated. After experimental intranasal influenza virus infection of mice, virus replication is known to be restricted primarily to the respiratory

epithelium (Eichelberger et al., 1991b) and is cleared within 10 days (Topham et al., 1997). It is therefore likely that a wide dispersal of activated lymphocytes is a characteristic of all effector cells and not simply restricted to what was observed in the murine influenza virus infection model. The widespread dissemination of antigen-specific $CD8^+$ T cells would be expected to have important consequences for protection from secondary infection, and therefore it would appear advantageous for primary and memory $CD8^+$ T cells to undergo this widespread dispersal following any infection.

Following the clearance of infection, the immune response was observed to establish memory populations which were found to be circulating in the spleen, MesLN, MedLN and the D-NALT. As previously discussed it seems likely that the memory population identified in the D-NALT would have an effector memory phenotype. These cells are resident in a prime location for contact with antigen and activation upon secondary infection, and this would be a desirable immune response for a vaccine to generate.

It was hoped that IEF analysis of fragment cultures would elucidate additional information regarding the humoral immune response with regard to the clonality and migration of response generated. It is suggested that this type of analysis should elucidate information regarding the ideal strength and anatomical location of an AFC response that would be desirable to be induced by vaccination. Unfortunately, time constraints required that the investigation moved away from this type of analysis. A novel method for analysis of the serum humoral response following infection was however developed.

Following a detailed characterisation of the immune response mediated by influenza virus infection, this study went on to characterise the response

generated by vaccination of influenza virus NP DNA using a ballistic device. Initially, one immunisation of two non-overlapping doses of DNA (approximately 0.5μg each) was administered and the virus-specific immune response characterised by IFNγ ELISPOT assay. Unfortunately the single immunisation regimen that was adopted in these initial studies did not appear to stimulate a detectable serum or mucosal virus or NP-specific antibody response. ELISPOT data showed a predominance of the virus-specific cellular response to be located in the spleen 14 days post immunisation. A significant response was also observed in the lymph nodes draining the immunisation site (IngLN) as well as the MesLN and more surprisingly the D-NALT. It is believed that this is the first report of a mucosal response initiated in the D-NALT by a single gene gun immunisation of plasmid to an abdominal site. The response generated was similar in size and kinetics when compared to that induced by influenza virus infection. There was however one striking difference in the responses observed in the D-NALT, and that was the ability of influenza virus infection to generate a memory population observed six months post infection. It is likely that this could be an important correlate of protection from further infection. Ideally any novel vaccine candidates should induce a similar memory population that would be expected to correlate with longevity of vaccine efficacy.

The generation of a memory response at potential sites of infection is a very desirable characteristic which may aid in the rapid mobilisation of a protective immune response following secondary encounter with antigen. The generation of a robust memory response has been linked to the magnitude of the initial primary response in an infection (Hou et al., 1994). It would appear that the generation of a substantial primary response is reflected in an enhanced memory established thereafter. Recently, research in the field of DNA vaccinology has focused on improving gene expression in the hope of

enhancing antigen expression and generating a correspondingly higher primary response and increased development of a memory cell population. Codon optimisation is a technique recently adopted to enhance protein expression by increasing the translational efficiency of a gene. Many pathogens have a different codon bias compared to mammals. This bias can result in low expression levels of pathogen's genes in transfected mammalian cells and this may correlate with low immunogenicity of some potential DNA vaccines. In such cases, it can be beneficial to synthesise genes containing the natural amino acid sequences but using the mammalian preferred codons for the amino acids. Several studies on codon optimisation have been carried out on the expression of human immunodeficiency virus (HIV) and human papillomavirus (HPV) proteins and have shown enhanced expression of, and improved immune response to target antigen (Casimiro et al., 2002, Cid-Arregui et al., 2003, Liu et al., 2004, Mossadegh et al., 2004, Ramakrishna et al., 2004). Another common method of enhancing the primary immune response to DNA vaccination is that of the use of genetic adjuvants. This involves the use of genes encoding cytokines, growth factors or co-stimulatory molecules which are included with the gene of interest in the same plasmid, or administered on a separate plasmid premixed with the intended immunising plasmid (Chattergoon et al., 2004, Eo et al., 2001b, Miyahira et al., 2003, Moore et al., 2002).

When PMID was assessed for production of a humoral immune response it was concluded that a single immunisation was insufficient to generate an antibody response. There are a few reports that have demonstrated that a single DNA immunisation is inadequate for the generation of a humoral immune response and it often takes several immunisations or a prime boost regimen to generate a significant antibody response (Johnson et al., 2000, Kasinrerk et al., 2002). It is likely that this could also be the case in this study. Unfortunately time constraints

limited any further investigation into the generation of a humoral response. It was never assumed that a single immunisation would be sufficient to mimic the natural response generated by influenza virus infection and obviously this is another major difference between experimental viral infection and immunisation that would have to be addressed should this technique and plasmid be considered for further studies.

When efforts concentrated on characterising the response following DNA prime and influenza virus challenge, it was initially observed that the majority of the influenza virus-specific $CD8^+$ IFNγ secreting cells produced were rapidly recruited to the lungs. As a result of enumerating the titre of virus in the lungs at this stage following challenge, it was discovered that the cell infiltration correlated with reduced viral replication. Viral titres were observed to be 2 logs lower compared with titres recovered from lungs of control mice immunised with empty vector before subsequent influenza virus challenge. Mice primed with pVac-NP were observed to clear the virus faster from their lungs and it was hypothesised that the reduction in viral replication was as a result of the IFNγ being produced in the lungs. To clarify this observation, attempts were made to deplete (*in vivo*) the IFNγ secreting cells that were thought to contribute to the reduction in lung viral titre. Unfortunately, at the timepoint chosen to analyse lung homogenates for virus titre, some of the mice had cleared virus from their lungs and no firm conclusions could be made regarding the effects of depletion of $CD8^+$ T cells.

An alternative approach to depleting $CD8^+$ T cells *in vivo* using monoclonal antibody would be to investigate influenza virus infection in mice with a disrupted β2-microglobulin (β2-M) gene. β2-$M^{-/-}$ mice lack MHC class I restricted $CD8^+$ T cells, and in the influenza mouse model they have been shown to have a higher

mortality rate to infection. Functional $CD8^+$ T cells are necessary for survival when the virulent influenza virus A/PR/8/34 is used to challenge mice (Bender et al., 1992). $\beta2\text{-}M^{-/-}$ mice would be an ideal strain to investigate the role of $CD8^+$ T cells following PMID of influenza virus NP DNA and subsequent viral challenge. Adoptive transfer of $CD8^+$ T cells from $\beta2\text{-}M^{+/+}$ mice would show the central role that $CD8^+$ T cells play in influenza virus infection.

Although protection was observed following PMID of influenza NP DNA, there was still a residual level of influenza virus replication in the lungs. Perhaps if an increased humoral response had been stimulated following the initial DNA immunisation it would enhance efficacy by neutralising virus particles. This would require further characterisation of the immunisation regimen, and assessment of the best method to obtain a strong antibody response. It could be possible that two or three immunisations could generate a humoral response. Alternatively, a prime boost regimen, using DNA in combination with either protein or with a recombinant viral vector expressing influenza virus NP could be a method to consider. Prime boost combinations have been shown to dramatically enhance protective immune responses generated in several mouse and primate models (McShane, 2002, Santra et al., 2004, Tritel et al., 2003, Wang et al., 2004). However, systemic prime-boost regimens do not always generate good immunity at mucosal sites. Recently it has been shown that mucosal prime-boosting immunisations can successfully generate mucosal and systemic responses (Eo et al., 2001a, Gherardi et al., 2004, Goonetilleke et al., 2003, Makitalo et al., 2004). Perhaps a mucosal prime-boost regimen would be the optimal method to use in the further development of this study. A recent study has described M cell targeted immunisation using a reovirus protein which binds to the surface of M cells in the NALT (Wu et al., 2001). When conjugated to DNA, this protein was found to induce prolonged mucosal IgA production and

an enhanced cellular CTL response. There is also evidence that DNA plasmids can be engineered to enhance their capacity to elicit humoral responses but this may compromise their ability to generate good cellular responses e.g. the use of secretory signals can enhance release of a protein and generate better humoral responses while maintenance of an intracellular antigen correlates with good Class I presentation and induction of cellular responses (Dr Fiona Cook, personal communication).

The final objective of this study was to characterise the influenza virus-specific effector $CD8^+$ T cells further, with respect to their anatomical location, expression of homing molecules and migratory preferences. Following intranasal influenza virus infection $CD8^+$ $tetramer^+$ cells were assessed for their expression of CD44, CD11a, CD62L and CD69. Throughout the tissues examined over a 35 day period following infection, the activated virus-specific $CD8^+$ T cells were found to have similar expression levels of these molecules over time and were predominantly of the $CD44^{hi}CD11a^{hi}CD62L^{lo}$ phenotype. Lymphocytes isolated from the lungs and draining lymph nodes (MedLN) were also found to be $CD69^{hi}$. The presence of CD69 on T cells is usually considered to reflect triggering via the TCR (Cosulich et al., 1987, Ziegler et al., 1994b) and in the influenza model, stimulation with antigen (Lawrence & Braciale, 2004). However, at day 35 post infection, these cells were still $CD69^{hi}$, at a time when PCR studies have shown that the influenza virus genome in no longer present. Influenza virus genome has not been detected more than 14 days post infection (Eichelberger et al., 1991b) and analysis of APC distribution following respiratory virus infection has shown that APCs are not observed after day 9 post infection (Usherwood et al., 1999b).

During influenza virus infection, there is a massive clonal expansion in the number of virus-specific $CD8^+$ T cells (Tripp et al., 1995), but most of the cellular progeny are destined to die by Fas-mediated mechanisms as the infection is resolved (Topham et al., 1997). Despite the high rate of apoptosis, some virus-specific $CD8^+$ T cells survive and continue to be retained in the lung. Is it possible that the persistent high expression of CD69 is a characteristic of terminally differentiated $CD8^+$ T cells destined for apoptosis? A recent publication has shown that that the majority of $CD8^+$ T cells isolated from non-lymphoid tissues during acute and memory responses to influenza virus infection express the $\alpha_1\beta_1$ intergrin (VLA-1). Additionally, on lung $CD8^+$ lymphocytes, VLA-1 expression was found to correlate to resistance from the apoptosis that follows recovery from influenza virus infection (Ray et al., 2004). Perhaps further analysis of CD69 expressing virus-specific lung lymphocytes for coexpression of VLA-1 could explain the continued expression of the CD69 activation antigen in the absence of influenza virus.

When the virus-specific $CD8^+$ T cells generated following PMID immunisation were analysed, they were found to have a similar $CD44^{hi}CD11a^{hi}CD62L^{lo}$ phenotype. Lymphocytes isolated from the IngLN, which drain the site of immunisation (the skin), were found to have a similar $CD69^{hi}$ phenotype as was observed in the lungs and MedLN following influenza virus infection. This extended expression of CD69 could perhaps be explained in this case by the continued presence of antigen. It is possible that NP gene expression could be maintained longer than influenza virus can be detected in the lungs following experimental infection. One of the original DNA vaccination studies reported the expression of protein, from a plasmid injected intramuscularly, to continue for greater than 1 year (Wolff et al., 1992) which could explain and extended expression of CD69.

From these data, it was observed that both intranasal influenza virus infection and PMID immunisation of DNA both induced virus-specific effector cells that have a similar phenotype with regard to their expression of CD44, CD11a and CD62L. Only considering these adhesion molecules it seems that DNA immunisation has generated a similar primary response compared to that generated by experimental infection. However, expression of several other adhesion molecules could be examined as potential candidates whose expression may correlate with immune protection. This study briefly described the problems of pentamer staining and subsequent analysis of the expression of the $\alpha_4\beta_7$ integrin on the lymphocytes analysed. One of the initial aims was to characterise the expression of this α_4 integrin on the D-NALT cells, as expression of this antigen has been associated with mucosal homing of lymphocytes (Hamann et al., 1994). It would have been interesting to see if the α_4 integrin was also expressed by lymphocytes isolated from the D-NALT and whether we could establish a similarity between lymphocytes found in the gut following influenza virus infection and those located in the D-NALT. Are these cell populations derived from the same population of activated effector cells, or do the D-NALT cells represent a specialised population? Taking into account the intermediate expression of CD44 on the lymphocytes isolated from the D-NALT recorded following both influenza virus infection and PMID immunisation of DNA, it suggests that these cells are specialised in some way characterised by a unique level of CD44 expression.

Further analysis of activated virus-specific cells involved a study of their homing characteristics. Total $CD8^+$ T cells were isolated from the spleens of mice either intranasally infected with influenza virus or immunised by PMID with NP DNA, before the transfer of these cells into naïve recipients to characterise their homing preferences. In both instances, the cells were observed to home to non-

lymphoid tissues, as recently described (Masopust et al., 2004). It was however observed that virus-specific cells had a preference to home to the respiratory mucosal tissues.

Studies have been carried out to investigate lymphocyte homing to the ONALT or the inductive site of the NALT (Csencsits et al., 1999, Csencsits et al., 2002). Within the ONALT, it has been shown that all HEVs express peripheral lymph node addressin (PNAd). Anti – L-selectin (CD62L) monoclonal antibody has been shown to prevent >90% of naïve lymphocyte binding to ONALT HEVs, whereas anti-MAdCAM (which blocks almost all naïve lymphocyte binding to PP HEVs) minimally blocks binding. This suggests a greater role in the ONALT for L-selectin – PNAd interactions, rather than $\alpha_4\beta_7$ – MAdCAM-1 interactions associated with the gut mucosa. It has been shown that $\alpha_4\beta_7$ – MAdCAM-1 interactions do not play a major role in lymphocyte homing to the lung or pulmonary tissues (Abitorabi et al., 1996, Picker et al., 1994), but do provide a mechanism for protective immunity against gut pathogens (Williams et al., 1998). It is therefore accepted that the $\alpha_4\beta_7$ – MAdCAM-1 interaction is regarded as intestinal rather than mucosal.

This study focussed on the effector site of the NALT, the DNALT. It is possible that homing to the DNALT is controlled in a similar manner as to that of the ONALT but this has still to be elucidated.

The techniques of IEF and flow cytometry were used to characterise anti-viral lymphocytes following influenza virus infection and vaccination in the hope that information regarding their location and phenotype could establish information about their trafficking by virture of the CMIS. IEF failed to generate the required clonal information due to a lack of sensitivity and time constraints. The analysis

of influenza virus-specific $CD8^+$ T cells however, showed no major difference in the phenotype of cells isolated from various tissues of the immune system following either influenza virus infection or PMID of NP DNA. This result could indicate the circulation of virus-specific lymphocytes throughout the immune system via the CMIS. However, it would be unfeasible to assess the expression levels of all adhesion molecules involved in lymphocyte homing, so no firm conclusions can be made. The homing characteristics of splenic $CD8^+$ T cells were assessed following intranasal influenza virus infection and PMID of NP DNA. From this data, it would appear that recently activated virus-specific $CD8^+$ T cells are free to traffic to the effector sites of the respiratory immune system, regardless of the method of their activation.

From all of the data generated in this study the most important correlates of protection that an influenza vaccine should induce are:

- A virus-specific neutralising antibody response
- A virus-specific IFNγ secreting $CD8^+$ T cell response in the lungs
- A memory virus-specific cellular response in the D-NALT
- Activated virus-specific T cells with a $CD44^{hi}CD11a^{hi}CD62L^{lo}$ phenotype

Analysis of PMID as a method of DNA vaccination has shown that it is capable of inducing a mucosal virus-specific response in the D-NALT, and that it is an effective method for inducing protection using the NP gene as a model antigen. However, no evidence was obtained to show that the method can induce a memory T cell population in the D-NALT and in comparison with the experimental infection, a substantial cellular response was not observed in the lungs following a single immunisation. More importantly there was no stimulation of an antigen-specific antibody response. By chance however, a response was

observed in the lung following two immunisations administered one week apart (the immunisation regimen adopted for the transfer studies) supporting the idea that a prime boost regimen dramatically enhances an immune response. This regimen of immunisation could also potentially induce an antibody response, however this was not assessed in this study. A single PMID immunisation appears to be an efficient method of influenza virus NP immunisation that could be adapted to generate a better vaccine. The response that it failed to generate in the lungs was resolved by giving two immunisations and it is possible that codon optimisation could increase gene transcription ultimately increasing the primary immune response and subsequent establishment of memory.

Future Directions

Several interesting results have been generated in this study and reviewing recent literature has prompted further questions that would have been interesting to address had time allowed. To offer a more complete set of data, I would initially envisage this study continuing by revisiting the $CD8^+$ depletion experiment following DNA prime and influenza virus challenge. It would be crucial to analyse lung homogenates at an earlier timepoint post challenge, before the NP vaccinated individuals clear the virus. It would also be interesting to analyse the effect of $CD4^+$ T cell depletion, and the combination of $CD4^+$ and $CD8^+$ T cell depletion. Secondly, it would be interesting to compare the homing of activated virus specific cells induced by intramuscular DNA immunisation to establish whether the method of PMID immunisation generates cells that have a preference to home to the D-NALT or whether it is a characteristic of DNA immunisation in general.

Thinking of a more long term analysis, I would finally suggest the following routes of investigation:

- Phenotypic analysis of virus-specific cells induced by influenza virus infection and PMID immunisation with regard to their expression of the integrins $\alpha_1\beta_1$ (implied in immune protection from influenza virus infection), $\alpha_4\beta_1$ (with its link to CD44 expression) and $\alpha_4\beta_7$ (for its role in mucosal homing).
- Analysis of two PMID immunisations of DNA one week apart and their ability to enhance both humoral and cellular immune responses.
- And finally, the effects of a prime boost regimen, perhaps including a recombinant virus expressing influenza virus NP as a mucosal prime or boost.

Chapter 7

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